

**EMBO GLOBAL LECTURE COURSE AND
SYMPOSIUM ON AMEBIASIS: EXPLORING THE
BIOLOGY AND THE PATHOGENESIS OF *Entamoeba***

ABSTRACT BOOK

DATE: 4TH- 7TH MARCH, 2012

VENUE: KHAJURAHO, INDIA

SPONSORS

JAWAHARLAL NEHRU UNIVERSITY (JNU)

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PROGRAM SCHEDULE

Day 1, March 4th, 2012

13:00	Arrival Khajuraho
16:00-16:20	Inauguration of the Conference Alok Bhattacharya, India
16:20-17:00	David Mirelman, Weizmann Institute, Israel Modulation of gene expression in <i>Entamoeba histolytica</i> : a short review of successes and failures.
17:00-17:20	Coffee Break
17:20-19:20	Session I - Genomics and transcriptomics Chairperson: Graham Clark, UK
17:20-17:50	Neil Hall, Institute of Integrative Biology, University of Liverpool, UK Genomic Diversity of the human gut parasite <i>Entamoeba histolytica</i>
17:50-18:20	Chung-Chau Hon, Pasteur Institute, Paris Characterization of a complex Repertoire of rare splicing isoforms, Endogenous Small RNAs and natural Antisense RNAs in <i>Entamoeba histolytica</i> using High-throughput sequencing.
18:20-18:50	Sudha Bhattacharya, School of Environmental Sciences, JNU, India Novel features of ribosomal DNA transcription and SINE mobilization in <i>Entamoeba histolytica</i> : an overview.

- 18:50 -19:05** **Ian Wilson , Institute of Integrative Biology, University of Liverpool,UK**
Annotation of the draft *Entamoeba moshkovskii* (strain Laredo) genome assembly
- 19:05-19:20** **Vijay Pal Yadav, School of Environmental Sciences, Jawaharlal Nehru University, India**
Study of induced retrotransposition in a competent cell line of *Entamoeba histolytica*
- 19:30** **Social mixer and dinner**

Day 2, March 5th, 2012

- 8:45-11:00** **Session II- Cell biology**
Chairperson: Cecilia Ximenez, Mexico
- 8.45-9.25** **Plenary Lecture**
Nancy Guillen, Pasteur Institute, Paris
Endoplasmic Reticulum stress-sensing mechanism is activated in *Entamoeba histolytica* upon treatment with nitric oxide.
- 9.25:-9:55** **A. Bhattacharya, School of Life Sciences, JNU**
Actin dynamics and signaling system in *Entamoeba histolytica*
- 9:55-10:25** **I Meza, CINVESTAV-IPN, Mexico**
Molecular and functional characterization of an *Entamoeba histolytica* protein EhMLC1 with features of a myosin essential light chain
- 10:25-10:45** **Swati Tiwari, School of Biotechnology, JNU, India**
Endoplasmic Reticulum Associated Degradation pathway for quality control of membrane and secretory components in *Entamoeba histolytica*

- 10:45-11:00** **Somlata, School of Life Sciences, JNU, India**
- C2 domain containing protein kinase is involved in initiation of phagocytosis in
protozoan parasite *Entamoeba histolytica*
- 11:10-11:30** **Coffee Break**
- 11:30-1:10** **Session III - Molecular biology**
Chairperson: Michael Duchene, Austria
- 11:30-12:10** **Plenary Lecture**
Upinder Singh, Stanford University, USA
Small RNA and gene regulation in *Entamoeba histolytica*
- 12:10-12:40** **Serge Ankri, Technion, Faculty of medicine, Israel**
The *E. histolytica* methyl LINE- binding protein EhMLBP serves as a bridge between
environmental stress response and epigenetic regulation.
- 12:40-13:10** **Luis Briebe, CINESTAV-IPN, Mexico**
Entamoeba histolytica contains a minimal scaffold to study DNA ligation
- 13:10:-15:30** **Session IV**
Lunch and poster session
Posters 1-28
- 15:30-17:30** **Visit to Temple**
- 17:30-18:00** **Coffee Break**

18:00-20:00 Session V Biochemistry

Chairperson: Neil Hall, UK

18:00-18:40 Plenary Lecture

Tomo Nozaki, National Institute of Infectious Diseases, Tokyo

Mitosome trafficking hydrolase carrier/receptor, metabolism and drug resistance in
Entamoeba histolytica

18:40-19:10: Michael Duchene, Institute of Specific Prophylaxis and Tropical Medicine, Austria

Entamoeba histolytica and metronidazole: 45 years of desperate amoebae and no
resistance in sight.

19:10-19:40: S. Gourinath, School of Life Sciences, JNU, India

Investigations into the loss of regulation in cysteine biosynthetic pathway of.
E. histolytica.

20:30 onwards- Dinner

Day 3, March 6th, 2012

6:00-9:30 Visit to Panna Tiger Reserve and Breakfast at Ken River Lodge

10:30-13:30 Session VI - Pathogenesis/Immunology

Chairperson: Sharon Reed, USA

10:30-11:10 Plenary Lecture

Kris Chadee, University of Calgary, USA

Entamoeba histolytica induces an acute pro-inflammatory response with increased
colonic permeability and altered tight junction proteins in Muc2^{-/-} mice

11:10-11:30 Coffee Break

- 11:30-12:00** **Iris Bruchhaus, Bernhard Nocht Institute for Tropical Medicine, Germany**
Influence of cysteine peptidases on abscess formation of *Entamoeba histolytica*
- 12:00-12:20** **Kumiko Nakada-Tsukui, National Institute of Infectious Diseases, Japan**
A novel class of cysteine protease receptors that mediate lysosomal transport
- 12:20-12:50** **Elisabetha Labruyere, Pasteur Institute, Paris**
Impact of collagen fibres networks on *Entamoeba histolytica* invasive process of the human colon mucosa
- 12:50-13:10** **Young Ah Lee, Yonsei University, South Korea**
Cysteine protease activity of *Entamoeba histolytica* is closely involved in amoebic adherence and host cell apoptosis.
- 13:10-13:30** **Katherine Ralston, University of Virginia, USA**
Chew your food: partial ingestion may play a role in host cell killing by *Entamoeba histolytica*.
- 13:30-16:00** **Session- VII**
Lunch and poster session
Posters 29-57
- 16:00-19:10** **Session VIII- Host response, Diagnosis, Epidemiology**
Chairperson: Isaura Meza, Mexico
- 16:00-16:40** **Plenary Lecture**
William Petri, University of Virginia, USA
Genome-wide sh-RNA screen identifies human host factors crucial for *Entamoeba histolytica* cytotoxicity
- 16:40-17:00** **Coffee Break**

- 17:00-17:30** **Graham Clark, London School of Hygiene and Tropical Medicine, UK**
The continuously expanding universe of *Entamoeba*
- 17:30-18:00** **Carol Gilchrist, University of Virginia School of Medicine, USA**
A Multilocus sequence Typing system (MLST) reveals a high level of diversity and a genetic component to *Entamoeba histolytica* virulence.
- 18:00-18:30** **Cecilia Ximenez, CINESTAV-IPN, Mexico**
Cutaneous amebiasis: the importance of molecular diagnosis in an emerging parasitic disease
- 18:30-18:50** **Jaishree Paul, School of Life Sciences, JNU, India**
Interaction of host and parasitic factors in the outcome of amoebiasis
- 18:50-19:10** **Sanghamitra Raha, Bose Institute, India**
Role of Mitogen activated kinase (EhMAPK) in stress survival of *Entamoeba histolytica*
- 20:00 onwards** **Dinner**

Day 4, March 7th, 2012

- 9:00-10:30** **Session IX - Vaccines, Drug development**
Chairperson: Serge Ankri, Israel
- 9:00-9:30** **Sharon Reed, University of California, USA**
Thioredoxin Reductase: A New Drug Target for Treatment of Amebiasis
- 9:30-9:50** **Amir Azam, Jamia Milia Islamia University, India**
Synthesis, Characterization and anti amebic properties of some heterocyclic compounds

9:50-10:10	Anjan Debnath, University of California, USA Hsp90 inhibitors as new leads to target parasitic diarrheal diseases
10:10-10:30	Hanna Lotter, Hamburg, Germany Contribution of host immune cells to the tissue destruction during amebic liver abscess development
10:30-11:00	Conclusion and summary Chairperson: Egbert Tannich, Germany
11:00	Coffee and Departure

Oral presentations

Modulation of gene expression in *Entamoeba histolytica*: a short review of successes and failures

David Mirelman¹ and Rivka Bracha¹

Dept. of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

The ability to modulate the expression levels of selected genes is one of the most effective ways to investigate their role in the different functions of a living cell. Thanks to the efforts of the groups of Drs. Petri and Tannich, who were the first to demonstrate in 1995 that episomal transfection could be used to express an exogenous gene in trophozoites of *E. histolytica*, we all know today much more on how to stably up- and down-regulate the expression of numerous amoebic genes. Such manipulations of individual genes have contributed a lot to our better understanding of their role in important aspects of the parasite such as in its metabolism, virulence and regulation of its life cycle. Nowadays a multitude of transfectants with different phenotypic modifications are available in the community of investigators and may be used for further studies. Unfortunately, we still lack a genetic system for *E. histolytica* and despite a lot of efforts by many investigators; we still do not know how to integrate exogenous DNA into the amoeba genome. These limitations have hampered our ability to perform genetic crosses and molecular manipulations such as gene knock-outs or knock-ins which are basic molecular tools that have been successfully used for many years in other organisms.

Genomic diversity of the human gut parasite *Entamoeba histolytica*

Gareth D Weedall¹, C Graham Clark², Kanok Preativatanyou¹, Pia Koldkjaer¹, Suzanne Kay¹, Iris Bruchhaus³, Egbert Tannich³, Steve Paterson¹ and Neil Hall¹

¹Institute of Integrative Biology, University of Liverpool, Biosciences building, Crown Street, Liverpool L69 7AH, UK

²Pathogen Molecular Biology Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

³Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany

Although we now have a good picture of the genome structure of *Entamoeba histolytica* little is known about the genetic diversity of the parasite. We re-sequenced the genomes of ten laboratory cultured lines of the eukaryotic pathogen *Entamoeba histolytica* in order to develop a picture of genetic diversity across the genome.

We were able to define putative single nucleotide polymorphisms throughout the genome. We found evidence indicating that recombination has occurred in the history of the sequenced genomes, suggesting that *E. histolytica* may reproduce sexually. We will present analysis of specific SNP loci using PCR based genotyping assays.

Using these data in combination with divergence data between *E. histolytica* and the related species *Entamoeba dispar* allowed us to identify candidate genes that may be evolving under positive natural selection. Genes and gene families with putative roles in virulence are among the more polymorphic genes. Large differences in coverage depth among genes indicate differences in gene copy number between genomes.

Our Analysis suggests that recombination is important factor in population structure of *E. histolytica* and that copy number variation may also be an important source of genetic variation between strains.

Characterization of a Complex Repertoire of Rare Splicing Isoforms, Endogenous Small RNAs and Natural Antisense RNAs in *Entamoeba histolytica* Using High-throughput Sequencing

Christian Weber,^{1,2} Odile Sismeiro,³ Caroline Proux,³ Sarbasshis Das,⁵ Mridula Agrahari,⁴ Jean-Yves Coppee,³ Alok Bhattacharya,^{4,5} Marie-Agnes Dillies,³ Bernd Jagla,³ Nancy Guillen,^{1,2,6} and Chung-Chau Hon,^{1,2,6}

¹ Institut Pasteur, Unité Biologie Cellulaire du Parasitisme, F75015, Paris, France; ²INSERM U786, F75015, Paris, France; ³ Institut Pasteur, Genopole, Transcriptome and Epigenome Platform, F75015, Paris, France; ⁴ School of Life Sciences, ⁵ School of Computational and Integrative Sciences, Jawaharlal Nehru University, New Delhi, India.

⁶ Corresponding authors

To characterize the largely unexplored transcriptome of the unicellular parasite *Entamoeba histolytica*, we performed a comprehensive census on its RNA populations using high throughput sequencing. In this study, we focused on 4 aspects, including gene model revision, alternative splicing, antisense transcripts and small RNAs. First, we revised and validated ~85% of the existing gene models. Then, we systematically analyzed the splicing events and reconstructed the alternative splicing isoforms on a genome-wide scale. We demonstrated most of the rare alternative splicing events are, in general, likely to be derived from inherent error of the constitutive splicing events (i.e. noisy splicing). Next, we demonstrated the pervasive existence of antisense transcripts and provided evidences to support most of them are not likely to originate from promiscuous leaky transcription of neighboring genes, but rather likely to originate from the 3' end of their sense counterpart. Lastly, we characterized a complex repertoire of small RNA populations, which has 5' monophosphate, enriched at 27nt with starting nucleotide of G, and mapped to exons of coding genes. We then demonstrated the differential expressions of mRNA between two strains are negatively correlated with that of the antisense small RNAs, suggesting the potential roles of these small RNAs in regulation of gene expression. In conclusion, our study is considered as the first extensive census on the RNA populations in *E. histolytica*, providing valuable resources for the research community and representing a major leap forward in the genome biology of the parasite since its genome was sequenced.

Novel features of ribosomal DNA transcription and SINE mobilization in *Entamoeba histolytica*: an overview

Sudha Bhattacharya

School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067

Protozoan parasites have revealed various novel phenomena like trans splicing of leader sequences, extensive mRNA editing and antigen switching, and are thus valuable systems to study basic biological processes. Here we present two novel observations from our studies with *E. histolytica* which add to the information gained so far from studies with model organisms. The first observation pertains to the regulation of rRNA transcription during growth stress. We present evidence to show that in *E. histolytica* rRNA synthesis does not stop under growth stress. Instead the pre rRNA accumulates along with a class of small RNAs (0.7-0.9 kb) derived from the 5'- external transcribed spacer (ETS). Further we show that the 5'-ETS RNAs, which are otherwise destined for degradation, are stabilized due to their ability to self-circularize. The role of these small RNAs in regulating pre rRNA processing under stress is being explored.

The second observation relates to the mode of retrotransposition in *E. histolytica*. It is well known that the genome of this organism harbors non long terminal repeat retrotransposons of which the autonomous members are called LINEs and the non autonomous members are called SINEs. Although LINEs are present in hundreds of copies, most of these are mutated and do not encode the functions required for retrotransposition (e.g. reverse transcriptase and endonuclease). We have generated a cell line of *E. histolytica* which encodes these functions on a plasmid and is thus retrotransposition-competent. We have studied the mobilization of a marked SINE copy in this cell-line and show that SINEs are indeed retrotransposed in these cells and that SINE chimeras are formed at high frequency as a consequence of retrotransposition. The possible mechanisms that generate these chimeras will be discussed.

Annotation of the draft *Entamoeba moshkovskii* (strain Laredo) genome assembly

Wilson, Weedall and Hall

Institute of Integrative Biology, University of Liverpool, Liverpool, UK

Entamoeba moshkovskii has widely been considered a free-living, non-pathogenic species. It will grow at room temperature and has been isolated from environmental sources. However, several studies have found *E. moshkovskii* infections associated with symptomatic amoebiasis, suggesting that it may be infective and pathogenic. Comparative genomic studies of *E. moshkovskii*, the pathogenic *Entamoeba histolytica* and the putatively non-pathogenic *Entamoeba dispar* may identify genomic features that shed light on *E. moshkovskii*'s lifestyle. Recent studies have identified differences in the species' genomes, such as the absence of variable short tandem repeats from tRNA gene arrays in *E. moshkovskii*. Here, the draft annotated genome of *E. moshkovskii* Laredo is presented. 454 reads were assembled de novo using Newbler software, with a peak alignment depth of 27x. A training set of 197 manually defined *E. histolytica* orthologues was used to predict gene models throughout the genome. 15,711 putative genes were identified, though this is likely to be significantly reduced through manual curation of the genome. The draft of the *E. moshkovskii* Laredo genome has been made publically available via the amoebaDB web resource and annotation will also be made available to aid genomic studies of these parasites.

Study of induced retrotransposition in a competent cell line of *Entamoeba histolytica*

Vijay Pal Yadav¹, Ashwini Kumar Ray¹, Alok Bhattacharya², Sudha Bhattacharya¹

¹School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India

²School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

The transposable elements are diverse, abundant and ubiquitous components of eukaryotic genomes and have been classified in two broad groups i.e. DNA transposons and retrotransposons. The Non-long terminal repeat (LTR) retrotransposable elements constitute ~11% of the 23 Mb genome of *Entamoeba histolytica*. The autonomous and non-autonomous non-LTR retrotransposons in this protist are classified in three families of each and referred as long (LINEs) and short interspersed nuclear elements (SINEs). EhLINE1 encodes two non-overlapping ORFs i.e. ORF1 and ORF2 which provides functions needed for retrotransposition. SINEs utilize enzymatic machinery encoded by LINEs for their retrotransposition. The ORF2 contains centrally located reverse transcriptase (RT) domain and C-terminal endonuclease (EN). Although EhLINE1 element is present in few hundred copies per genome, no single active copy has been identified since most of them have accumulated mutations including large deletions. In order to study active retrotransposition we have created *E. histolytica* cell line expressing ORF2 protein from an inducible episomal construct with constitutive endogenous expression of ORF1 protein and a marked-EhSINE1 expressing from another construct. We observed that this marked-EhSINE1 was inserted in the provided target site upon expression of ORF2 protein. Insertion was accompanied by target site duplication- a hallmark of retrotransposition. In addition to marked-EhSINE1, we detected other copies of SINEs which displayed sequence polymorphism similar to polymorphism seen in the genomic copies. This system exactly mimicked the retrotransposition process of *E. histolytica* and is an excellent model system to understand the detailed mechanism of retrotransposition and propagation and evolution of these elements in this protist. In addition to this we analyzed the sequence mosaicism in the genomic copies of SINEs and correlated with the sequence polymorphism obtained, experimentally.

Endoplasmic reticulum stress-sensing mechanism is activated in *Entamoeba histolytica* upon treatment with nitric oxide

Julien Santi-Rocca^{1,2}, Sherri Smith^{1,2}, Christian Weber^{1,2}, Erika Pineda³, Chung-Chau Hon^{1,2}, Emma Saavedra³, Alfonso Olivos-García⁴, Sandrine Rousseau⁵, Marie-Agnès Dillies⁶, Jean-Yves Coppée⁶ and Nancy Guillén^{1,2}

¹Institut Pasteur, Unité Biologie Cellulaire du Parasitisme, Paris, France

²INSERM U786, Paris, France

³Instituto Nacional de Cardiología, Departamento de Bioquímica, México DF, México

⁴Universidad Nacional Autónoma de México, Facultad de Medicina, México DF, México

⁵Institut Pasteur, Bioinformatics Group for Genomics analysis, Paris, France

⁶Institut Pasteur, Genopole, Transcriptome and Epigenome Platform, Paris, France

The Endoplasmic Reticulum is a dynamic organelle and changes in its size and components have been described, either as a result of changes in the secretory capacity of cells or as a result of adaptation to diverse stresses. Stressing the ER prompts a cell response known as the unfolded protein response (UPR), which restores normal ER function and prevents the cytotoxic impact of malformed proteins. The *Entamoeba histolytica* endomembrane system is simple compared to those of higher eukaryotes, as a canonical ER is not observed. Our precedent work suggests that virulence factors of this parasite are related to the expression of stress response genes (1) and should be linked to UPR activation upon exposure of parasites to tissue inflammation factors (e.g. ROS, NO, cytokines). In this work we address the question of how this ancient eukaryote responds to stress induced by immune components (i.e. NO) and whether stress leads to ER changes and subsequently to an UPR. Using live imaging and confocal microscopy we found that NO dramatically provokes extensive ER fragmentation which appears as a protective response against stress. Profiling gene expression patterns during incubation with NO revealed that stress is marked by (i) dramatic up-regulation of hsp genes although a bona fide UPR is absent; (ii) induction of DNA repair and redox gene expression and iii) up-regulation of glycolysis-related gene expression. Enzyme activity measurements demonstrate that NO directly inhibits glycolysis and consequently explains the decrease of ethanol and ATP levels (2), which in conjunction with ER fragmentation increase parasite death. The link between virulence and stress response was then more deeply explored by RNA deep-sequencing of *Entamoeba histolytica* displaying diverse levels of virulence. Our data show a clear correlation between the increase of virulence and the enhancement of a stress response (3).

1. Santi-Rocca et al., 2008. Cell Microbiol 10: 202-217
2. Ramos E et al., 2007. Exp Parasitol 116: 257-265
3. Weber et al., 2012. Poster session in this symposium

This work is supported by to NG from the Pasteur-Weizmann Research Council and from the French National Research Agency and to SS from the Pasteur Foundation.

Actin dynamics and signaling system in *Entamoeba histolytica*

Alok Bhattacharya¹, Somlata¹, Saima Aslam¹, Mohd Shahid Mansuri¹, Mrigya Babuta¹, Ruchi Jain^{1*}, Narendra Padhan^{1*}, Nivedita Sahoo^{1*} and Sudha Bhattacharya²

¹School of Life Sciences and ²School of Environmental Sciences, Jawaharlal Nehru University, New Delhi

*Currently different affiliation

Genome sequencing and subsequent analysis suggested that *E. histolytica* is likely to have extensive signaling network. Genes encoding two classes of molecules, trans membrane kinases (TMK) and calcium binding proteins (CaBPs) were the dominant signaling molecules that were found in the *E. histolytica* proteome and their presence suggested that this organism is likely to have novel signaling pathways. Majority of TMKs and CaBPs did not have any close homologs in any known organism based on sequence comparison. We have systematically investigated the signaling systems that involve TMKs and CaBPs. Our results show novel pathways and functions associated with these molecules. For example, we show that CaBP1 and CaBP3 along with a protein kinase Ehak1 are involved in actin dynamics and consequently participate in actin mediated processes, such as phagocytosis and pseudopod formation. We also present data to show that B1 family of TMKs are involved in cellular proliferation and that lipids can activate the expression of TMKB1-9 through inositol triphosphate kinase pathway.

Molecular and functional characterization of an *Entamoeba histolytica* protein (EhMLCI) with features of a myosin essential light chain

Meza I,¹ Díaz-Valencia DJ,¹ Franco E,¹ Benítez-King G,² Villegas-Sepúlveda N,¹

¹Departamento de Biomedicina Molecular. Centro de Investigación y de Estudios Avanzados del IPN, Apartado 14-740, México DF, 07360, México. ²Instituto Nacional de Psiquiatría, Departamento de Neurofarmacología, Subdirección de Investigaciones Clínicas, México DF, México.

Entamoeba histolytica, a protozoan parasite of humans, relies on its striking motility to survive and invade host tissues. Characterization of the molecular components involved in motile processes is crucial to understand its pathogenicity. Although protein components of myosin II hexamers have been predicted from *E. histolytica* genome data, only a heavy chain of myosin, EhmhcA, has been characterized so far. We have cloned an *E. histolytica* cDNA sequence that best matched *Dictyostelium discoideum* myosin essential light chain and found that the cloned sequence is transcribed as an mRNA of 0.445 kb which could encode a protein of 16.88 kDa, within the predicted range for a myosin light chain. In silico analyses revealed that the protein sequence, named EhMLCI, shows two consensus domains for binding MHC, but lacks the N-terminal sequence for actin binding, as in A2 type myosin essential light chains. A single EF-hand calcium-binding domain was identified in the C-terminus and several high score predictability sites for serine and tyrosine phosphorylation. Antibodies to recombinant EhMLCI identified two proteins of approximately 17 and 15 kDa in trophozoite extracts, the latter phosphorylated in tyrosines. Serine phosphorylation was not detected. Immunomicroscopy revealed EhMLCI cortical and cytoplasmic distribution in trophozoites and true colocalization with EhmhcA determined by PCC. Co-immunoprecipitation corroborated EhMLCI interaction with EhmhcA. EhMLCI was also localized in actomyosin-containing complexes. Differential partition of phospho-tyrosinated EhMLCI into cell fractions containing the soluble form of EhmhcA and its lack of serine phosphorylation suggest its possible participation in a novel down regulatory mechanism of myosin II activity in *E. histolytica*.

Endoplasmic Reticulum Associated Degradation pathway for quality control of membrane and secretory proteins in *Entamoeba histolytica*

Preeti Gupta and Swati Tiwari

School of Biotechnology, Jawaharlal Nehru University, New Delhi, India

Entamoeba histolytica adheres to host colonic epithelial cells and secretes proteases and porin-forming peptides. These functions are crucial to its fitness as a parasite. In order to ensure the quality of secretory and membrane proteins, eukaryotic cells have evolved mechanisms that ensure that misfolded proteins do not enter the secretory pathway and are degraded by ubiquitin-proteasome mediated degradation in a process termed as Endoplasmic Reticulum Associated Degradation (ERAD). ERAD is linked to the Unfolded Protein Response (UPR) pathway whose purpose is to alleviate stress of endoplasmic reticulum (ER). Since N-linked glycans and quality control machinery in lower eukaryotes is not identical to those of higher eukaryotes, it is likely that the rules governing ERAD and UPR in these organisms are different from those in higher eukaryotes.

We have identified homologs of two ER-associated ubiquitin conjugating enzymes (E2s), Ubc6 and Ubc7 from higher eukaryotes in *E. histolytica*. These genes (EhUBC6 and EhUBC7) were cloned and expressed as fusion proteins in *E. coli*. EhUbc7 formed thioester bond with ubiquitin showing that it is a biochemically active E2 enzyme. Replacement of conserved cysteine at position 92 (Cys92) to serine in EhUbc7 abolished this activity indicating that Cys92 is the active site cysteine. The protein was found to be associated with ER by confocal immunofluorescence microscopy and subcellular fractionation. Treatment of trophozoites with tunicamycin that induces ER-stress by preventing N-linked glycosylation, resulted in upregulation of EhUbc7 transcript by several folds. This suggests that both UPR and ERAD are linked in amoeba and EhUbc7 plays a role in ERAD. Overexpression of dominant negative form of EhUbc7 (EhUbc7C₉₂→S) in *E. histolytica* resulted in an inhibition of phagocytosis of human RBCs. This suggests that EhUbc7 may modulate functioning of the secretory pathway in this organism.

C2 Domain Containing Protein Kinase is Involved in Initiation of Phagocytosis in Protozoan Parasite *Entamoeba histolytica*

Somlata¹, Sudha Bhattacharya² and Alok Bhattacharya^{1,3}

¹School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India,

²School of Environment Sciences, Jawaharlal Nehru University, New Delhi-110067, India.

³School of Computational & Integrative Sciences, Jawaharlal Nehru University, New Delhi-110067, India

Phagocytosis is a process whereby particles are taken in by cells through mechanisms superficially similar to those for endocytosis. It serves a wide range of functions, from providing nutrition in unicellular organisms to initiation of both innate and adaptive immunity in vertebrates. In the protozoan parasite *Entamoeba histolytica*, it has an essential role in survival and pathogenesis. Our laboratory is interested in deciphering the mechanisms of initiation of phagocytosis in *E. histolytica*. Phagocytosis is initiated when particles bind to receptors on the membrane of phagocytes. Cytoplasmic Ca²⁺ and a number of cytoskeletal proteins and their regulators, including actins, myosins, p21 activated kinases and Rho/Rac small GTPases, and the ARF family GTPases ARF1 and ARF6 are known to participate in the process. Phagocytosis in *E. histolytica* is likely to follow a different molecular path as many of the known players of the mammalian system have not been identified in this organism. Although much progress has been made in deciphering the pathways leading to phagocytosis in different organisms including *E. histolytica*, the early events of the process remain to be discovered. Here, we show that EhC2PK, a C2-domain-containing protein kinase, and the Ca²⁺ and actin-binding protein, EhCaBP1, are involved in the initiation of phagocytosis in *E. histolytica*. Conditional suppression of EhC2PK expression and overexpression of a mutant form reveals its role in the initiation of phagocytic cups. EhC2PK binds phosphatidylserine in the presence of Ca²⁺ and thereby recruits EhCaBP1 and actin to the membrane. Identification of these proteins in phagocytosis is an important step in amoebic biology and these molecules could be the important targets for developing novel therapies against amebiasis.

Small RNA and gene regulation in *Entamoeba histolytica*

Upinder Singh,

School of Medicine, Stanford University, USA

The *E. histolytica* methyl line binding protein EhMLBP serves as a bridge between environmental stress response and epigenetic regulation.

Sophia Katz, Oded Kushnir, Ayala Tovy, Rama Siman Tov, and Serge Ankri

Department of Molecular Microbiology, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, P.O.B 9649, 31096 Haifa, Israel.

Adaptation to environmental stress is a key process that allows the unicellular parasite *Entamoeba histolytica* to survive in its human host. We previously characterized EhMLBP as an essential protein for the growth and the virulence of the parasite. EhMLBP binds to methylated repetitive DNA, and is one of the core proteins of the parasite's epigenetic machinery. Here, we show that EhMLBP and heat shock proteins have common properties. EhMLBP is induced by heat shock and its expression is regulated by a heat shock element binding site that is located in its 5' non-coding region.

Following heat shock, EhMLBP displays an enhanced recruitment to the reverse transcriptase of a long interspersed nucleotide element (LINE) DNA. Constitutive overexpression of EhMLBP leads to an enhanced transcription of RT LINE and protects trophozoites against heat shock and reduces protein aggregation. Furthermore, upon heat shock EhMLBP is able to bind polyubiquitinated proteins. The protective function of EhMLBP to heat shock and its ability to bind polyubiquitinated proteins is lost in trophozoites that overexpress a mutated form of EhMLBP which is devoid of its heat shock domain. Following heat shock, the perinuclear localization of EhMLBP in control trophozoites is replaced by an even distribution within the nucleus and with the appearance of cytoplasmic vesicles. The disappearance of these vesicles in cyclohexamide-treated trophozoites and their induction in trophozoites that overexpress an amebic homolog of TIA-1, an RNA binding protein that promotes the assembly of stress granules, suggest that these vesicles correspond to stress granules.

In addition, we will present first evidences of an interaction between EhMLBP and the DNA/tRNA specific methyltransferase EhMeth that takes place under heat shock condition.

To the best of our knowledge, this is the first report of a methyl DNA binding protein that plays a protective role against heat shock.

***Entamoeba histolytica* contains a minimal scaffold to study DNA ligation**

Cesar Cardona, Samuel Lara¹, Elisa Azuara², Luis Briebe¹

1 National Laboratory of Genomics-Cinvestav-IPN, Irapuato Mexico

2 Posgrado en Ciencias Genómicas, Autonomous University of Mexico City, Mexico

DNA ligases are ubiquitous proteins involved in sealing the gaps during Okazaki fragment maturation and DNA repair. Vertebrates contains three kinds of DNA ligases, whereas protozoan from the genera *Plasmodium* or *Entamoeba* only contain one kind, dubbed type I. DNA ligase I from *E. histolytica* has a reduced amino acid length in comparison to DNA ligase of *H. sapiens*. This reduction in amino acid length is due to the lack of an extended 200 amino acid N-terminal region. We have characterized DNA ligase I from *E. histolytica* and shown that this ligase is an ATP-dependent DNA ligase that is expression is up-regulated by UV light and demonstrated its nuclear localization by confocal microscopy. Biochemical studies indicate that DNA ligase I from *E. histolytica* is quite promiscuous when it encounters and oxidative of UV DNA lesion, but it is quite selective for canonical DNA bases. Biochemical studies using this “minimal” ligase demonstrated a direct interaction between Proliferating Cellular Nuclear Antigen (PCNA) and DNA ligase I. The crystal structure of EhPCNA indicates a series of changes in the surface interaction area in comparison to PCNA from *H. sapiens*. Thus, the interaction between PCNA and DNA ligase I is due to a specific surface interaction between both proteins and is not only mediated by an interacting peptide. Our studies indicates that basic scientific questions can be solved using “minimal” proteins from protozoan parasites

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Novel transport machinery of the mitosome outer membrane in *Entamoeba histolytica*

Takashi Makiuchi¹, Yoshitaka Murakami^{1,2}, and Tomoyoshi Nozaki^{1,2}

¹ Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan, ² Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

The mitochondrion is the ubiquitous organelle in all living eukaryotes. Under microaerophilic or anaerobic conditions, however, it has evolved into highly divergent forms in structure, composition, and function. A few groups including ours have previously shown that *Entamoeba histolytica* has such a highly diversified form of the mitochondrion called mitosome, whose primary function is sulfate activation (Mi-ichi Proc Natl Acad Sci 2009; Mi-ichi PLoS NTD 2011). While the novel functions of mitosomes have started unveiled, the evolution of its import machinery for mitosomes is poorly understood. The *E. histolytica* genome encodes only two homologs of the outer membrane components: transporter of outer membrane (Tom) 40, a core component of the translocation channel, and Sam50. Using the biochemical approaches, we isolated and characterized a novel 600-kDa TOM complex, which contains a novel component, Tom60. When gene expression of Tom40 or Tom60 was repressed, the transport of mitochondrial proteins to mitosomes was inhibited, which resulted in severe growth defect. The phenotype was similar to that shown by repression of Cpn60 or enzymes involved in sulfate activation. These data suggest that Tom40 and Tom60 are essential for mitochondrial transport and cell proliferation. Tom60 possesses the tetratricopeptide repeats (TPR), like Tom20 and Tom70, which are membrane proteins and serve as a receptor to bind the mitochondrial targeting sequence. Surprisingly, Tom60 does not possess the transmembrane spanning region, and is distributed to both the cytosol and mitosomes. We also demonstrated that Tom60 binds to We propose that soluble cytosolic Tom60 binds to the precursor of mitochondrial proteins, and tows them to the outer membrane of mitosomes.

***Entamoeba histolytica* and metronidazole: 45 years of desperate amoebae and no resistance in sight.**

David Leitsch, Sarah Schlosser, Julia Matt, Margit Tazreiter, Marina Binder, Michael Duchêne

Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria

Metronidazole chemotherapy of *E. histolytica* has now been used for 45 years, nevertheless, many questions remain about its interaction with the parasite. By two-dimensional gel electrophoresis, microarray hybridizations, and qPCR we studied the response of *E. histolytica* to metronidazole. Surprisingly, no large changes in mRNA or protein expression were observed, however, activated metronidazole formed covalent adducts with thioredoxin, thioredoxin reductase, superoxide dismutase, and purine nucleoside phosphorylase. A similar small set of specific target proteins was also modified in *Trichomonas vaginalis* and *Giardia intestinalis*. Our data are in contrast to the view that metronidazole indiscriminately damages proteins. Thioredoxin reductases from all three parasites catalyzed the NADPH-dependent metronidazole reduction, and proteins known to interact with the thioredoxin system were covalently modified by activated metronidazole. In *E. histolytica*, we identified more thioredoxin interaction partners by means of a method using mutated recombinant thioredoxin as a bait. Recombinant thioredoxin and thioredoxin reductase from *E. coli* were also modified by metronidazole, and had significantly diminished disulfide-reducing activity. Although there is only a limited number of target proteins of the thioredoxin system, it is difficult to find those most relevant for metronidazole activity. So, in another approach, we study the downstream effect of DNA degradation, to show the possible contribution of parasite DNases.

Fortunately, *E. histolytica* has not developed clinically relevant resistance. The highest concentration of 40 μ M metronidazole to which the amoebae could be adapted (Wassmann et al., 1999) is far below the drug levels in the patient. In these amoebae, thioredoxin reductase was down-regulated. Metronidazole-resistant *T. vaginalis* had normal levels of thioredoxin reductase, which was inactive due to a lack of flavin cofactor. In *G. intestinalis*, resistant strains also lacked reduced flavin. Taken together, thioredoxin reductases are important both for metronidazole activation and resistance.

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Investigations into the loss of regulation in cysteine biosynthetic pathway of *E. histolytica*

Sudhir Kumar¹, Isha Raj¹, Isha Nagpal¹, N. Subbarao² and S. Gourinath¹

School of Life Sciences¹, School of computational and integrative sciences², Jawaharlal Nehru University, New Delhi-110067, India

Cysteine (cys) plays a major role in growth and survival of the human parasite *Entamoeba histolytica*. The crystal structure of serine acetyltransferase (SAT) isoform1, a cysteine biosynthetic pathway enzyme, from *E. histolytica* (EhSAT1) at 1.77 Å, in complex with its substrate serine (ser) at 1.59 Å and inhibitor cys at 1.78 Å resolution are determined. EhSAT1 exists as a trimer both in solution as well as in crystal structure, unlike hexamers formed by other known SATs. The difference in oligomeric state is due to the N-terminal region of the EhSAT1, which has very low sequence similarity to known structures, also differs in orientation and charge distribution. The ser and cys bind to the same site, confirming that cys is a competitive inhibitor of ser. The disordered C-terminal region and the loop near the active site are responsible for solvent accessible acetyl Co-A binding site and thus lose inhibition to acetyl Co-A by the feedback inhibitor cys. Docking and fluorescence studies show that EhSAT1 C-terminal mimicking peptides can bind to o-acetyl serine sulfhydrylase (EhOASS), while native C-terminal peptide does not show any binding. To test further, C-terminal end of EhSAT1 was mutated and found that it inhibits EhOASS, confirming modified EhSAT1 can bind to EhOASS. The apparent inability of EhSAT1 to form a hexamer and differences in C-terminal region are likely to be the major reasons for the lack of formation of the large cysteine synthase complex and loss of a complex regulatory mechanism in *E. histolytica*.

Thioredoxin Reductase: A New Drug Target for Treatment of Amebiasis

Larissa Podust¹, Anjan Debnath¹, Derek Parsonage², Ken Hirata³, Leslie Poole², James McKerrow¹, and Sharon L. Reed³

¹Department of Pathology, University of California, San Francisco School of Medicine, San Francisco, CA, USA, ²Department of Biochemistry, Wake Forest School of Medicine, Winston-Salem, NC, USA, ³Departments of Pathology and Medicine, University of California, San Diego School of Medicine, San Diego, CA, USA

The majority of patients with amebiasis worldwide are treated with a single class of drugs, the 5-nitroimidazoles, particularly metronidazole. Although metronidazole is very effective for acute amebiasis, finding additional drug targets is important for such a significant cause of morbidity and mortality. We earlier reported the high throughput screening assays of 13,000 compounds against *E. histolytica* trophozoites. One of the active compounds was auranofin, an FDA-approved oral gold compound. Based on expression arrays of the genes up- and down-regulated in trophozoites following auranofin treatment and known auranofin targets, we identified thioredoxin reductase as the most likely drug target. Auranofin had an IC₅₀ of 0.4 μ M against active, recombinant *E. histolytica* thioredoxin reductase (EhTrxR). Since *Entamoeba histolytica* does not have a glutathione reductase system, EhTrxR is critical for protecting amebic trophozoites from oxidant attack. When trophozoites were exposed to auranofin (2 μ M) for 18 hours, they became more sensitive to killing by H₂O₂, had increased intracellular reactive oxygen species detected with dichlorodihydrofluorescein, and the intracellular thioredoxin was predominantly in the oxidized state.

The thioredoxin reductase of *E. histolytica* is similar to *E. coli* TrxR, and neither contain selenium found in most eukaryotic thioredoxin reductases. To understand the interaction of auranofin with EhTrxR, recombinant EhTrxR was expressed in *E. coli*, purified, and crystallized after reduction with NADPH and in the presence of gold chloride and auranofin. The gold from gold chloride and auranofin bound most tightly to Cys 286, which is also present in the *Giardia* TrxR. *Giardia* is also susceptible to auranofin, including metronidazole-resistant strains. These findings suggest that thioredoxin reductase is a valid drug target in *E. histolytica*, which can be inhibited with a “repurposed” FDA approved drug, auranofin.

***Entamoeba histolytica* induces an acute pro-inflammatory response with increased colonic permeability and altered tight junction proteins in Muc2^{-/-} mice**

Vanessa Kissoon-Singh, France Moreau, Kris Chadee

Gastrointestinal Research Group, Department of Microbiology, Immunology & Infectious Diseases, University of Calgary, Calgary, Alberta

Human colonic MUC2 mucin is the first line of innate host defense against enteric pathogens and in preventing *E. histolytica* induced epithelial injury. *E. histolytica* (Eh) binds the mucus layer via the parasite surface Gal-lectin to mucin Gal and GalNAc oligosaccharides for colonization, and to the colonic epithelium to initiate invasion. Mice deficient in Muc2 spontaneously develop colitis and are more susceptible to chemical and bacteria-induced injury. In this study, we quantified the early innate immune responses toward Eh colonic challenge in Wt and Muc2 deficient mice. Virulent Eh trophozoites challenged in colonic loops of Wt but not Muc2^{-/-} animals induced a time-dependent significant increase in mucin and non-mucin glycoprotein secretion as revealed by ³H-glucosamine metabolic labeling studies. Immunohistochemical staining revealed intense and sustained Muc2 mucin secretion in Wt mice that formed a thick, protective mucus blanket overlying the surface epithelium and entrapping Eh in a mucous exudate. Surprisingly, in Muc2^{-/-} animals, Eh did not bind or invade the colonic epithelium devoid of mucus, but was present away from the epithelium in a viscous secretory exudate dominated by increased serum albumin leakage. Moreover, in Muc2^{-/-} animals, Eh induced a significant intense time-dependent watery secretory response that correlated with increased gross pathology scores as compared to Wt animals. Colonic pathology and secretory responses were dominated by increased TNF- α and IFN- γ protein secretion that correlated with altered expression of the tight junction proteins claudin-2 and occludin. Among the putative various virulent factors tested, Eh cysteine protease 5 (EhCP5) was the major virulence factor that elicited pro-inflammatory responses and aberrant protein secretions in the colon. We conclude that colonic Muc2 mucins confer both luminal and epithelial barrier functions and in the absence of Muc2, animals are more susceptible to Eh-induced secretory and pro-inflammatory responses mediated by EhCP5.

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Influence of cysteine peptidases on abscess formation of *Entamoeba histolytica*

Jenny Matthiesen, Ann-Katrin Bär, Dennis Marien, Laura Biller, Hannelore Lotter, Egbert Tannich, Iris Bruchhaus

Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

On certain occasions the trophozoites of *Entamoeba histolytica* penetrate the intestinal wall leading to invasive diseases like ulcerative colitis or extra-intestinal abscesses. The mechanisms that allow *E. histolytica* to invade and induce abscesses are not completely understood. We identified two syngenic cell lines derived from the widely used laboratory *E. histolytica* strain HM-1:IMSS which constantly differ in pathogenicity. Cell line A is incapable to induce liver abscesses, whereas cell line B is able to induce large abscesses. In vitro studies indicate that cell line B has a 10 times higher cysteine peptidase activity compared to the non-pathogenic cell line A. The aim of our work is to clarify if a certain *E. histolytica* cysteine peptidase (EhCP) of the papain-like EhCP family, consisting of 35 members, plays a decisive role in abscess formation. In this context, we found in studies using mice and gerbils as models for amoebic liver abscess (ALA) formation, that the expression of seven ehcp genes is upregulated in trophozoites directly isolated from ALA compared to axenically cultured trophozoites. To test the influence of these CP's on the pathogenicity, transfectants were generated that specifically overexpress the CP's of interest in the non-pathogenic cell line A. ALA-formation was induced by injection of the respective transfectants into the liver of mice and abscess formation was evaluated. First results indicate that at least two of the EhCP-overexpressing transfectants are able to rebuild the pathogenic phenotype.

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A novel class of cysteine protease receptors that mediate lysosomal transport

Kumiko Nakada-Tsukui¹, Atsushi Furukawa^{1, 2}, Kumiko Tsuboi¹, Yoko Yamada¹, Tomoyoshi Nozaki^{1, 3}

¹Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan;

²Department of Microbiology, Graduate School of Medicine, Gunma University, Maebashi, Japan; ³Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan

The transport of lysosomal proteins is, in general, mediated by mannose-6-phosphate receptors, which recognize carbohydrate modifications of the cargo proteins. Here, we describe a novel class of receptors that regulate the transport of lysosomal hydrolases in *Entamoeba histolytica*. A 110-kDa cysteine protease (CP) receptor (CP-binding protein family 1, CPBF1) was initially discovered by affinity co-precipitation of the major CP (EhCP-A5), which plays a pivotal role in the pathogenesis of *E. histolytica*. We have demonstrated that CPBF1 regulates EhCP-A5 transport from the endoplasmic reticulum to lysosomes and its binding to EhCP-A5 is independent of carbohydrate modifications. Repression of CPBF1 by gene silencing led to the accumulation of the unprocessed form of EhCP-A5 in the non-acidic compartment and the mis-secretion of EhCP-A5, suggesting that CPBF1 is involved in the trafficking and processing of EhCP-A5. We also have demonstrated that other predominant CPBF proteins, CPBF6 and CPBF8, bind to amylases, and α -hexosaminidase and lysozymes, respectively. These results suggest that the CPBF represents a new class of transporters that regulate the trafficking, processing, and activation of lysosomal enzymes and, thus, regulate the physiology and pathogenesis of *E. histolytica*.

Impact of collagen fibers networks on *Entamoeba histolytica* invasive process of the human colon mucosa

Roman Thibeaux^{1,2}, Alexandre Dufour³, Pascal Roux⁴, Michèle Bernier⁵, Anne-Catherine Baglin⁵, Pascal Frileux⁶, Jean Christophe Olivo-Marin³, Nancy Guillén^{1,2} and Elisabeth Labruyère^{1,2}

¹ Institut Pasteur, Unité Biologie Cellulaire du Parasitisme, Paris, France,

² INSERM U786, Paris, France,

³ Institut Pasteur, Unité Analyse d'Images Quantitative, Paris, France,

⁴ Institut Pasteur, Imagopole, Plate-forme d'Imagerie Dynamique, Paris, France,

⁵ Hôpital Foch, Service Anatomopathologie, Suresnes, France

⁶ Hôpital Foch, Chirurgie générale et digestive, Suresnes, France

Our interest is to characterize *E. histolytica* factors and human components that could influence the migration of the parasite through the human colonic mucosa during intestinal amoebiasis. The aim of the present study was to characterize the architecture of the interstitial collagen fiber network in live human intestine and to determine whether migration of *E. histolytica* through the extracellular matrix was protease dependant or independent.

The behavior of *E. histolytica* wild type and silenced for the cysteine protease A5 (CP-A5) were compared on a three-dimensional collagen matrix and an ex vivo model based on human colon explants. Real time imaging (4D) using multiphotonic microscopy techniques combined to a second harmonic generation signal were used to visualize cells and collagen fibers without fluorescent probes. Parasite motility and morphology were quantified with a newly implemented 3D cell tracking software.

Our results reveal for the first time two different interstitial collagen fiber scaffolds within the colonic connective tissue. To penetrate the tissue, *E. histolytica* migrates on the dense scaffold that remained intact, reaches the crypt of Lieberkhün, migrates along and then disorganizes the loose scaffold to escape into the mucosa. Interestingly, *Entamoeba* collagenase activity is supported by secretion of cysteine proteases and although CP-A5 is not required in vitro for this activity, CP-A5 is absolutely necessary for collagen meshwork remodelling and subsequent invasion and thus, suggesting that CP-A5 has in vivo an indirect role on collagen network disorganization.

In this study, our data show that collagen fibers architectures impact *E. histolytica* invasive route and that further step of invasion relay with ECM destruction that requires human components induced or activated in the presence of CP-A5.

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Cysteine protease activity of *Entamoeba histolytica* is closely involved in amoebic adherence and host cell apoptosis

Young Ah Lee¹, Seong Hoon Kim¹, Arim Min¹, Kyoung-Joo Song¹, Yumiko Saito-Nakano², Tomoyoshi Nozaki², David Mirelman³, Myeong Heon Shin¹

¹Department of Environmental Medical Biology, Institute of Tropical Medicine, and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea.

² Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, Japan.

³Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

Entamoeba histolytica is an enteric tissue-invasion protozoan parasite that causes amoebic colitis and occasionally liver abscess in humans. Amoebic major virulence factors include Gal/GalNAc lectin, amoebapore, amoebic cysteine proteinases (CP). During tissue invasion, amoebic adhesion to host tissue components and host cell death is important events for successful infection. However, amoebic factors for adhesion to tissue components and host cell apoptosis are not fully understood. In this study, we compared the ability of amoeba to adhere to host factors or to kill host cells using various *E. histolytica* strains with mutations in major virulence factors. The adherent capacity of the amoebic Gal/GalNAc lectin-silenced strain L5 and the non-pathogenic Rahman strain, but not G3 strain deficient with amoebapore, was significantly reduced compared to the pathogenic wild-type *E. histolytica*. Interestingly, the Rahman strain exhibited decreased CP activity compared to the wild-type amoeba. Next, to address whether CP is responsible for amoebic adhesion to ECM protein, wild-type strain was pretreated with a CP inhibitor, CP modifier, or antibodies to specific CP. Pretreated amoeba resulted in a marked decrease in adhesion to ECM proteins. In addition, inhibitor of cysteine protease (ICP1)^{-/-} strain with hyper-CP secretion showed a significant increase in amoebic adherence to host components compared to control strain. In addition, ICP1 overexpressing amoeba with hypo-CP secretion exhibited reduced ability to adhere to ECM protein. Moreover, Rahman strain or ICP1 overexpressing amoeba, but not G3 strain, showed reduced ability to induce apoptosis or dephosphorylation in host cells such as Jurkat T or hepatocyte cells. These results strongly suggest that amoebic CP activity is important factor affecting amoebic adhesion to host tissues and host cell apoptosis, causing tissue inflammation induced by *E. histolytica*. This study will be useful to understand the cross talk between amoeba and host in amoeba-invaded lesions during human amoebiasis.

Chew your food: partial ingestion may play a role in host cell killing by *Entamoeba histolytica*

Katherine S. Ralston¹, Michael D. Solga², Alexandra M. Bettina² and William A. Petri, Jr.^{1,2,3}

¹ Department of Medicine, ² Department of Microbiology, and ³ Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, VA, USA

The pathogenesis of amoebiasis is associated with profound tissue destruction, manifesting as ulceration of the intestinal epithelium or abscesses in extraintestinal sites. The cytotoxic activity of the parasite is central to tissue destruction, but the exact mechanism by which host cell death is induced is unknown. We sought to elucidate the mechanism by first employing live cell fluorescence video microscopy to observe host-parasite interactions in real time. Surprisingly, we found that within one minute of interaction, the parasite internalizes distinct “pieces” of the targeted human cell. Ingested “pieces” are also seen within one minute by transmission electron microscopy. We examined markers for target cell viability, and found that this “partial ingestion” precedes target cell killing, as assessed by irreversible calcium elevation and membrane permeabilization, which occur within about three minutes and about fifteen minutes, respectively. Together, these data imply an unusual mechanism for cell killing, where partial ingestion is an early step that is later followed by cell death. We are currently employing multiple strategies to inhibit phagocytosis in order to determine if partial ingestion is absolutely required for host cell killing. Notably, by using Amnis Imagestream analysis to quantitatively examine ingestion over time, we have rarely detected complete internalization of the targeted human cell. Therefore we suggest that complete ingestion may not be the “goal” and that rather the “offensive” nature of partial ingestion represents a mechanism to elicit cell killing. Thus, through these studies we have uncovered surprising host-parasite interactions and are beginning to get a clearer picture of how this parasite effects such devastating tissue destruction.

Genome-wide shRNA screen identifies human host factors crucial for *Entamoeba histolytica* cytotoxicity.

Chelsea Marie, Hans P. Verkerke, Dan Theodorescu, William A. Petri, Jr.

Department of Medicine, Department of Microbiology, and Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, VA, USA

Human infection with *Entamoeba histolytica* depends on the parasites ability to kill human cells. Amebic cytotoxicity is dependent on host cellular signaling machinery but the precise mechanisms of cell death are not well understood. We used amebic cytotoxicity to select a human shRNA knockdown library of mammalian cells. Genomic DNA from successive rounds of selection was subjected to advanced sequencing to identify shRNA knockdowns with resistance parasite cytotoxicity. After nine rounds of selection only 434 genetic knockdowns remained in the library screened with a high ratio of parasite to host cell (1:5) and 812 remained in a library screened with a low ratio of parasite to host cell (1:50).

Several genes previously implicated in parasite cytotoxicity were identified by this method including pro-apoptotic and calcium channel genes. Novel targets of interest identified include kinases, surface receptors, sugar-modifying enzymes, and other ion channels genes. 100 genes were selected for secondary validation in an independent assay. Surprisingly 74% of the knockdowns selected in the high stringency screen were also independently selected in the low stringency screen. Based on these results, 100 genes were selected for secondary validation by esiRNA knockdown. 45 of these 100 genes were found to significantly reduce parasite cytotoxicity when knocked down in host cells, including several surface molecules and ion channel genes. To further resolve the functional relevance of ion channels as drug targets, we utilized a pharmacological approach by testing the ability of ion channel inhibitors to block amebic cytotoxicity in vitro. These results demonstrate proof-of-concept that whole genome shRNA screens can successfully identify druggable targets. The results of this work provide insight into the host factors that *E. histolytica* parasites exploit to kill mammalian cells and provide an extensive framework for further studies of host susceptibility to *E. histolytica*.

The continuously expanding universe of *Entamoeba*

C Graham Clark¹, Eloise J Busby¹, Alison S Shearer¹, Amparo Vidal-Lapiedra², C Rune Stensvold³

¹London School of Hygiene and Tropical Medicine, London, UK, ²University of Valencia, Valencia, Spain, ³Statens Serum Institut, Copenhagen, Denmark

In 1919 Dobell concluded that all the descriptions of *Entamoeba* in humans could be ascribed to three species: *E. histolytica*, *E. coli*, and *E. gingivalis*. At this time morphology and host were the primary bases for naming species. We now know that both are unreliable, since host ranges vary and identical morphology can hide substantial differences. The application of molecular tools, especially DNA sequencing, has greatly increased our understanding of variation within this genus but initial reliance on cultures gave us only a limited insight. For the past few years we have been using DNA extracted directly from faeces from a wide range of hosts to explore previously hidden *Entamoeba* diversity. Recently published data showed the existence of a uninucleate clade from non-human primates related to *E. bovis* and substantial diversity within *E. coli*. We also reported expanded host ranges for some species, with *E. coli* being found in a rodent and an *E. muris*-like organism (designated Entamoeba RL7) in a non-human primate from SE Asia. We have subsequently identified *Entamoeba* RL7 in children from Liberia, West Africa, expanding the diversity of human *Entamoeba* yet again. A new lineage distantly related to *E. muris* has been detected in a wild bank vole and a new lineage unrelated to *E. equi* has been detected in a horse, both in the UK. Finally, a new reptilian lineage has been found in an Aldabran tortoise in Mauritius. These results suggest that our picture of *Entamoeba* diversity is still very incomplete and further sampling is almost certain to uncover novel lineages. In particular, reptiles, amphibia and birds are very poorly sampled despite numerous species having been described in the older literature and deserve more attention.

A Multilocus Sequence Typing System (MLST) Reveals a High Level of Diversity and a Genetic Component to *Entamoeba histolytica* Virulence

Carol A. Gilchrist¹, IbneKarim M. Ali¹, Mamun Kabir², Faisal Alam³, Sana Scherbakova⁴, Eric Ferlanti⁴, Gareth D. Weedall⁵, Neil Hall⁵, Rashidul Haque², William A. Petri Jr.¹, Elisabet Caler⁴

Departments of Medicine, School of Medicine, University of Virginia, Charlottesville¹, Virginia, United States of America; International Centre for Diarrhoeal Diseases Research, Bangladesh²; Rajshahi Medical College, Rajshahi, Bangladesh³; J. Craig Venter Institute, Rockville, Maryland, United States of America⁴; Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom⁵.

The outcome of an *Entamoeba histolytica* infection is variable and can result in either asymptomatic carriage, immediate or latent disease (diarrhea/dysentery/amebic liver abscess). An *E. histolytica* multilocus genotyping system based on tRNA gene-linked arrays has shown that genetic differences exist among parasites isolated from patients with different symptoms. However, these arrays are highly variable among isolates from a restricted geographical range.

To identify genetic markers associated with clinical outcome we compared single nucleotide polymorphisms (SNPs) present in the publically available sequence data of 12 *E. histolytica* strains. We focused on SNPs, which in comparison with the sequence from the reference strain HM-1:IMSS, changed the encoded amino acid, and were present in independent *E. histolytica* isolates from different geographical origins. We designed specific primers to amplify the regions spanning the selected SNPs and tested 84 Bangladesh DNA samples obtained directly from stool and amebic liver aspirates and recently established xenic strains for the presence or absence of these SNPs. Each amplicon was linked to the original isolate sample source by a unique 7 base pair (bp) sequence “barcode”, combined with other samples, and sequenced in a single IlluminaGIIX lane. Using this technique a record of the SNPs present at 16 loci out of the original 21 selected targets was obtained for 44 DNA samples obtained directly from stool and amebic liver aspirates and 19 xenic *E. histolytica* strains.

Our work supports the previous finding of extensive haplotype diversity among *E. histolytica* isolates from the same geographic origin. Two SNPs at the cylicin-2 gene locus (EHI_080100) were significantly associated with amebic liver abscess samples and infrequent in diarrhea suggesting that SNPs at this locus are in linkage disequilibrium with a genetic trait associated with virulence and that there is a link between *E. histolytica* genetics and parasite virulence.

CUTANEOUS AMOEBIASIS: the importance of molecular diagnosis in an emerging parasitic disease.

Patricia Morán¹, René Cerritos¹, Liliana Rojas¹, Valeria Zermeño¹, Alicia Valadez¹, Miguel Angel Reyes², Griselda Montes de Oca³, Enrique González¹, Oswaldo Partida¹, Eric Hernández¹, Miriam Nieves¹, Tobías Portillo¹, Manuel Ramiro³, Cecilia Ximénez¹.

¹Department of Experimental Medicine, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, Mexico, ²Urology Department, General Hospital of Mexico, Ministry of Health, Mexico City; ³Dermatology Department, General Hospital of Mexico, Ministry of Health, Mexico City; ⁴Unit of Education, Scientific Research and Health Policy Direction of Medical Benefits, Mexican Institute of Social Security Mexico City, Mexico.

Cutaneous amoebiasis is the least common clinical form of human amoebiasis. Cases usually correspond to patients with deep skin lesions in areas such as; anal, perineal, vulvae, vaginal and genitalia, as well as the diaper area in babies with intestinal diarrhea due to *E. histolytica* infection. In adults the most common location of cutaneous amoebiasis is the abdominal area as a consequence of the abdominal wall spontaneous involvement of amoebic liver abscess or as a complication of an amoebic liver abscess drainage procedure. In Mexico this pathology was occasionally observed before the late 1980's. However, in the last decades, most of the documented cases of this condition around the world are among sexually transmitted amoebiasis cases. The cases refereed in the present work are of sexually transmitted genital amoebiasis. The molecular characterization of the *Entamoeba* species present in the affected tissues allows not only the deferential diagnosis of disease, but the documentation for the first time of a case of cutaneous amoebiasis where *E. histolytica* and *E. dispar* was detected. This finding underline the importance of an opportune etiological diagnosis using specific and sensitive techniques to avoid the rapid destruction of tissues and the irreversible sequels on the anatomy and function of affected organs. On the other hand, the detection of a new evidence of mixed infections (*E. histolytica* and *E. dispar*) in an invasive case of amoebiasis open new perspectives in the study of the extraordinary complex host-parasite relationship in amoebiasis.

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Interaction of host and Parasitic factors in the outcome of amoebiasis

Jaishree Paul¹, Anil Verma¹, Lakshmi Rani Iyer¹, Nishant Singh² and Sudha Bhattacharya²

¹School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067

²School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067

Entamoeba histolytica is a microaerophilic intestinal protozoan parasite and the causative agent of invasive amoebiasis (colitis and Amoebic liver abscess) that remains a significant cause of morbidity and mortality in developing countries. Excystation in the intestinal lumen produces trophozoites and colitis results when the trophozoite penetrates the mucus layer and damages intestinal tissues. The trophozoites proliferate in lumen and phagocytose resident flora.

In order to study the role of gut microbiota during proliferation of the parasite, we quantified the population of predominant bacteria like Bacteroides, Bifidobacterium, Ruminococcus, Lactobacillus, Clostridium leptum subgroup, Clostridium coccoides subgroup, Eubacterium, Campylobacter, Methanobrevibacterium smithii and Sulphur reducing bacteria (sulphate reducing gene) using genus specific primers in healthy (N=22) vs amebic patients (N=17) stool sample by Real-time PCR. Significant alterations observed in the concentration of some beneficial bacteria reveal their potential role during the disease.

Among the host factors, we investigated the genetic polymorphism in the Leptin receptor gene (rs1137101-Q223R) that has been observed in a suppressed state in malnourished children associated with amoebiasis. The distribution of Leptin receptor gene in ALA patients of Northern India vs. controls found to be significant when compared between wild and heterozygous or homozygous. Risk factors associated with this mutation has been studied.

When the parasitic factors were considered, we observed significant differences in MIC values towards metronidazole in clinical isolates of amebic patients compared with HM1 axenic strain. Expression profiling of the genes that are targeted towards metronidazole resistance was carried out in the above isolates. *E. dispar* isolates exhibited low level of expression of these genes indicating the lack of scavenging pathway. Immunolocalization studies using monoclonal antibody against peroxiredoxin gene during stress conditions suggest that the antioxidant peroxiredoxin is important for protection against oxidative stress thus facilitating invasion.

Role of Mitogen activated protein Kinase (EhMAPK) in stress survival of *Entamoeba histolytica*

Anupama Sardar Ghosh, Doel Ray, Suman Dutta and Sanghamitra Raha

Crystallography & Molecular Biology Division, Saha Institute of Nuclear Physics, 1/AF Bidhan nagar, Kolkata-700064, INDIA

As survival strategies of *Entamoeba histolytica*, a disease-causing parasite are vital for the propagation of the disease amoebiasis, we examined the effects of several stress conditions in *E. histolytica*. We determined that heat stress did not induce cell death in *E. histolytica* but hydrogen peroxide did. We also characterized the cell death mechanism of this parasite which resembled higher eukaryotic physiological cell death or apoptosis in some aspects. EhMAPK, the sole typical MAPK of the *Entamoeba* genome was earlier characterized by us. Now we have extended the study to illustrate the activation mechanisms of the EhMAPK such as phosphorylations at certain amino acid residues (Tyrosine and Threonine) in the TDY motif of the activation loop and correlated the activation of this enzyme with stress survival in this parasite. Site directed mutagenesis experiments were carried out to characterize its activation mechanisms namely phosphorylations on these residues. Also, the different stress conditions that activate or deactivate the enzyme was worked out. 2.0 mM H₂O₂ treatment induced dephosphorylation of EhMAPK and loss of kinase activity. In contrast, heat shock at 43°C or 0.5 mM H₂O₂ treatment augmented the phosphorylation status of EhMAPK and boosted the kinase activity of the protein. 2.0 mM H₂O₂ treatment lowered parasite viability significantly but *E. histolytica* viability was not affected by heat shock or 0.5 mM H₂O₂ treatment. An increased activity of EhMAPK in vivo in response to non lethal stresses and a decreased activity in response to lethal stresses indicate a possible involvement of EhMAPK in survival responses of the parasite exposed to different forms of stress. Further studies are needed to identify the substrates of this enzyme in this parasite.

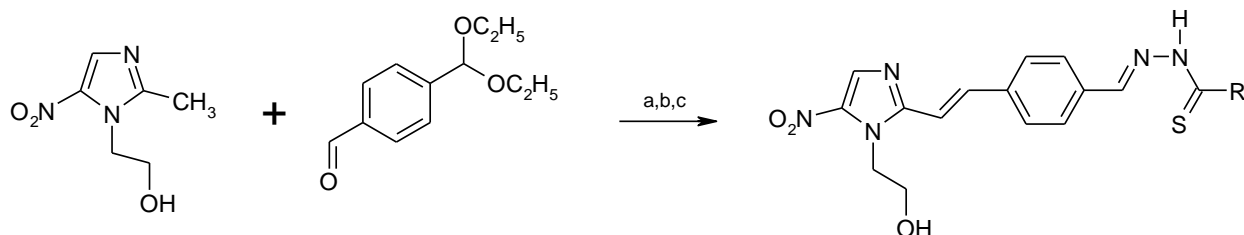
Therefore, a distinct possibility that activation of EhMAPK is associated with stress survival in *E. histolytica* is seen. Our study also gives a glimpse of the regulatory mechanism of the protein under in vivo conditions. Since the parasite genome lacks any typical homologue of mammalian MEK, the dual specificity kinases which are the upstream activators of MAPK, indications of the existence of some alternate regulatory mechanisms of the EhMAPK activity is perceived. These may include the autophosphorylation activity of the protein itself in combination with some upstream phosphatases which are not yet identified.

Synthesis, Characterization and Antiamoebic Activity of Some Heterocyclic Compounds

Amir Azam

Department of Chemistry, Jamia Millia Islamia University, Jamia Nagar, New Delhi-110025, India

Novel derivatives of Heterocycles such as Pyrazoline, Metronidazole thiosemicarbazone conjugates, dioxazole, Porphyrins, Hydrazone, Chalcone, Triazole embedded thiosemicarbazone and fused pyrazolo pyrimidinone were synthesized and evaluated for their in vitro antiamoebic activity and cytotoxicity, which displayed remarkable inhibition against HM1: IMSS strain of *E. histolytica*. Some metal complexes were also synthesized and characterized in order to find the effects of complexation on antiamoebic activity. The study suggests the beneficial potential of these leads that need to be further explored in order to develop better and safer therapeutic agents for amoebiasis.



Reagents and Conditions: (a) Sodium methoxide, DMSO, MeOH, rt; (b) HCl, THF, 50 °C ; (c) Ethanol, 80 °C, reflux.

Hsp90 inhibitors as new leads to target parasitic diarrheal diseases

Anjan Debnath¹, Dea Shahinas², Clifford Bryant³, Ken Hirata⁴, Grace Hwang⁴, Jiri Gut¹, Adam R. Renslo³, Dylan R. Pillai⁵, Sharon L. Reed⁴, and James H. McKerrow¹

¹Sandler Center for Drug Discovery, University of California, San Francisco, USA, ²Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada, ³Small Molecule Discovery Center, University of California, San Francisco, USA, ⁴Department of Pathology, University of California, San Diego, USA, ⁵Department of Pathology and Laboratory Medicine, University of Calgary, Alberta, Canada.

Entamoeba histolytica and *Giardia lamblia* are anaerobic protozoan parasites that cause amebiasis and giardiasis, two of the most common diarrheal diseases worldwide. Current therapy for amebiasis and giardiasis relies on metronidazole. Metronidazole has several adverse effects, and in vitro *E. histolytica* trophozoites easily adapt to therapeutically relevant levels. Drug resistance and recurrent infections are also common for giardiasis. Therefore, it is critical to search for effective and better-tolerated anti-amebic and anti-giardial drugs. As a first step in this effort, we have synthesized several Hsp90 inhibitors as leads for new chemotherapy. The most significant discovery is the amebicidal and giardicidal activities of an orally available Hsp90 inhibitor and two analogs. The inhibitors were re-screened to discriminate between amebicidal, giardicidal activities and general cytotoxicity towards a mammalian cell line. No mammalian cytotoxicity was found at >100 μ M for 48 h. The Hsp90 inhibitor has completed Phase I clinical trials in both solid and hematological tumor patients, is well tolerated in mice at 50 mg/kg 3 times a week for 3 weeks. In vivo efficacy of this Hsp90 inhibitor in a mouse model of amebic colitis documented significant inhibition of parasite growth at a single oral dose of 5 mg/kg/day for 7 days. To understand the mechanism of action of selected hits, a competitive binding assay was performed using the fluorescent ATP analogue bis-ANS and recombinant *E. histolytica* Hsp90. There was significant reduction in fluorescence compared to control, confirming *E. histolytica* Hsp90 as the target for this inhibitor. Considering in vitro activity and in vivo efficacy this Hsp90 inhibitor represents a promising therapeutic option for amebiasis and giardiasis.

Contribution of host immune cells to the tissue destruction during amoebic liver abscess development

Elena Helk¹, Hannah Bernin¹, Thomas Ernst², Harald Ittrich², Egbert Tannich¹,
Hannelore Lotter¹

¹Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany;

²Diagnostic and Interventional Radiology Department and Clinic, University Medical Center Hamburg-Eppendorf

Epidemiological and histological findings suggest that beside parasite specific effector molecules, immune pathological mechanisms might contribute to the development of amoebic liver abscess (ALA). ALA mainly occurs in male individuals and the abscessed liver areas are characterized by a massive infiltration of immune cells. Among these, neutrophils and macrophages bear the potential for tissue destruction by the release of effector molecules initially aimed to destroy the invading pathogen. We recently developed an immune competent mouse model that allows investigating immune pathological mechanisms underlying the development of ALA. By using a panel of neutralizing antibodies, respective knock-out mutant mice as well as pharmacological agents we selectively depleted neutrophils, monocytes as well as liver resident macrophages, the Kupffer cells. The impact of these cells on the development and the course of ALA were monitored by magnet resonance imaging (MRI). Immune depletion of neutrophils using the anti- Ly6G antibody resulted in a decrease in the ALA sizes compared to the size of ALA in wild-type (WT) control mice from day five following intrahepatic amoebic infection. Moreover, the use of anti-GR1 antibody that depletes neutrophils as well as macrophages significantly reduced the abscess sizes already from day one post infection. ALA sizes were also reduced in CCR2^{-/-} mice that are disabled in their ability to recruit macrophages to a site of infection. The elimination of liver resident macrophages by clodronate treatment again resulted in a significant reduction in the size of ALA compared to control mice. Unexpectedly, ALA sizes were not reduced in functional impaired iNOS-Knock-out mutant mice. From these studies we conclude that not only parasite effector molecules but also host immune mechanisms are responsible for the liver damage during ALA and the cell populations mainly responsible for tissue destruction belong to the macrophage lineage.

Poster Presentations

Circular transcripts from 5' ETS of pre rRNA with autocyclization property accumulate along with pre rRNA in stressed cells of *Entamoeba histolytica*

Abhishek Kumar Gupta¹, Alok Bhattacharya² and Sudha Bhattacharya¹

1 School of Environmental Sciences, Jawaharlal Nehru University, New Delhi-67, 2 School of Life Sciences, Jawaharlal Nehru University, New Delhi-67.

Entamoeba histolytica a protozoan parasite is the causative agent of amoebiasis, a food and water borne disease affecting about 50 million people worldwide. A unique feature of this parasite is that its rRNA genes are present exclusively on extra chromosomal plasmids, unlike other eukaryotes where these are present as tandem repeats on linear chromosomes. The rRNA genes of *E. histolytica* are organized as palindromic ribosomal DNA (rDNA) units (I and II) in a 24.5 Kb circle. We have earlier mapped the Transcription Start Points and promoters of the two rDNA units. Here we look at the effect of growth stress (serum starvation, cycloheximide treatment) on transcription of rDNA I. Contrary to expectation, the level of pre rRNA as measured by northern hybridization with External Transcribed Spacer (ETS) probe actually increased under stress, showing that rDNA transcription is not inhibited as confirmed by nuclear run on, but processing may get inhibited which leads to accumulation of unprocessed pre-rRNA. Accumulation of pre-rRNA is accompanied by accumulation of family of RNA molecules (912, 849, 766 and 666 nt) from distal part of 5'ETS called as ets RNAs. ets RNAs are not polyadenylated, lacks any ORF, are nuclear localized and are transcribed from the same strand as rRNA. etsRNAs are circular in nature and can spontaneously self circularize in vitro. This is unusual since the ETS part of pre rRNA is thought to be degraded after pre-rRNA processing. Since most of the ncRNAs have regulatory role these transcripts also may have role in regulation of rRNA synthesis or processing which remains to be explored in near future.

Amoebic intracellular hypoxia and heat shock protein response are required for *Entamoeba histolytica* pathogenicity and virulence

Alfonso Olivos-García¹, Mario Nequiz¹, Emma Saavedra², Nancy Guillén³, Nora Hernández³, Rusely Encalada², Erika Pineda², Erika Luis-García¹ and Ruy Pérez-Tamayo¹

¹Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F., México, ²Departamento de Bioquímica, Instituto Nacional de Cardiología Ignacio Chávez, México D.F., México. ³Institut Pasteur, Unité Biologie Cellulaire du Parasitisme, Paris, France

Symptomatic or asymptomatic intestinal human amoebic infections are partially explained by colonization of different strains of either non-pathogenic or pathogenic amoebae with variably virulent phenotypes. Several in vitro amoebic features, such as complement resistance, adhesion, cytotoxicity, proteolytic activity and phagocytosis, have been suggested as responsible for amoebic pathogenicity. Such features, however, are preserved when *E. histolytica* loses its ability to produce liver abscesses in hamsters due to prolonged in vitro culture (nvEh). Moreover, in contrast with virulent *E. histolytica* (vEh), non-pathogenic *E. dispar* and nvEh fail to survive beyond 24 h when injected into the hamster liver. During this limited time period amoebae are exposed to high O₂ concentrations, complement and ROS from inflammation. The role of the virulent parasite resistance to such stressing conditions on its pathogenicity is poorly understood. Using the amoebae mentioned above, we analyzed their susceptibility to physiological O₂ concentrations and under microanaerobic or supraphysiological O₂ environment: 1) the resistance to complement, O₂ and H₂O₂; 2) the O₂ and H₂O₂ reductive capacity; 3) the protein content of the NADPH flavin oxidoreductase and of the Hsp; 4) the degree of protection of Fe-S clusters in proteins and 5) the transcriptional responses of vEh and nvEh. Our results show that, in the three microorganisms examined, exposure to O₂ affects their Fe-S clusters of proteins (vEh<nvEh=*E. dispar*) and the complement resistance. Furthermore, *E. dispar* revealed the highest susceptibility to physiological O₂, complement and H₂O₂, which correlated with its lowest capacity to reduce O₂ and H₂O₂. On the other hand, nvEh showed a moderately decreased ability to reduce O₂ and H₂O₂, and absence of Hsp response, as compared with vEh. Our results suggest that both the amoebic intracellular hypoxia and its proper antioxidant defenses, are primary conditions for amoebic pathogenicity, and also that the Hsp response is required for virulence.

Molecular characterization of *Entamoeba histolytica* isolates using tRNA gene-linked short tandem repeats amplification in the United Arab Emirates.

Ali ElBakri¹, Amidou Samie², Sinda Ezzedine¹, and Raed Abu Odeh¹

¹ Department of Medical Laboratory Technology, College of Health Sciences, University of Sharjah, Sharjah, UAE; ² Department of Microbiology, University of Venda, Thohoyandou, South Africa.

Amoebiasis, caused by *Entamoeba histolytica*, ranges from the asymptomatic carrier state to the symptomatic state. Recently, the highly polymorphic short tandem repeats (STR) linked to tRNA genes have been shown to correlate with the virulence of individual *E. histolytica* strains. In the present study we aimed to investigate the genetic diversity of *Entamoeba histolytica* isolates among 19 asymptomatic expatriates from different geographic regions in Sharjah, the United Arab Emirates. Here, we evaluated the usefulness of two pairs of tRNA-linked STR loci using the RR and NK tRNA specific primers in identifying the polymorphism observed. As expected, a considerable degree of polymorphism was detected. Based on the PCR-size, fourteen genotypes were assigned from both loci combined. Moreover, no association between the different genotypes and the study population demographics was noted. The results demonstrate the extensive genetic variability among isolates from different geographic regions.

Functional characterization of ORF2 protein encoded by EhLINE1 in *Entamoeba histolytica*.

Amit Kumar Gaurav¹, Alok Bhattacharya² and Sudha Bhattacharya¹

¹School of Environmental Sciences, ²School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

Transposons constitute a large fraction of various genomes including human, yeast, plants, fungi and most of the protozoan parasites. Transposons have been grouped in two major classes, class-I retrotransposons and class-II DNA transposons. About 19.7% of the *E. histolytica* genome is constituted of transposons, majority of which are the non long terminal repeat (LTR) retrotransposons. These retrotransposons belong to multiple families of autonomous and non-autonomous elements called EhLINEs and EhSINEs respectively. The EhLINE1 element of *E. histolytica* encodes two ORFs (ORF1 and ORF2). The ORF2 contains a central reverse transcriptase (RT) domain and a C- terminal restriction enzyme like endonuclease (EN) domain. These domains of ORF2 are closely related to the R2 group of non-LTR Retrotransposons from *Bombyx mori*. We have studied the RT and EN activities of recombinant full length ORF2 protein. Wide distribution of EhLINE1 in the *E. histolytica* genome suggests that, unlike other members of R2 group of non LTR retrotransposons, the ORF2-EN may not be site specific. We show that the full length ORF2 protein has both EN and RT activity. As expected, the EN activity is not site-specific. We compare the processivity and fidelity of RT with RTs of retroviral origin.

Distribution of Q223R (Glutamine 223 arginine) Mutation in Leptin receptor of amoebic liver abscess(ALA) patients of North India

Anil Kumar Verma¹, Vineet Ahuja², Jaishree Paul¹

¹ School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. ² Department of Gastroenterology and HNU, All India Institute of Medical Sciences, New delhi, India.

Amoebiasis, a potentially fatal enteric infection caused by the protozoan parasite *Entamoeba histolytica* and amoebic Liver Abscess (ALA) results from extension of *E. histolytica* infection from the intestine to liver. Leptin is an adipocytokine produced by adipocytes that inhibits food intake, influences the immune system and is suppressed in malnourished children. Previous observations have shown that a genetic polymorphism (rs1137101-Q223R) in the leptin receptor affected susceptibility to *E. histolytica* infection and that leptin signaling has a protective role during intestinal amebic infection. Individuals segregating for the allele G which translate to arginine at position 223 of leptin receptor (LEPR), were found to be nearly 4 times more susceptible to have infection compared with those homozygous for ancestral allele A for glutamine (223Q). We investigated the prevalence of Q223R mutation in leptin receptor(LEPR) in ALA patients of north Indian origin. We collected 47 ALA pus Samples in sterile container from Gastroenterology department of AIIMS under supervision of a Gastroenterologist and 102 blood samples were collected as control from healthy individuals. DNA from ALA pus samples were extracted using Qiagen stool kit. Primers were designed from exon-6 of leptin receptor gene covering the Q223R mutation and PCR-RFLP was done with Restriction enzyme BseNI. Genotype frequency of alleles AA, AG and GG in control samples were 0.12, 0.56 and 0.32 whereas in ALA patients these were 0.15, 0.62 and 0.24 respectively. Allele frequencies for allele A and G in control were 0.40 and 0.60 whereas it was 0.46 and 0.54 in ALA patients. In Indian population Q223R mutation is significant when we compared wild(AA) with heterozygous(p=0.049) and homozygous mutant (p=0.004) genotype. This is the first report on Indian population on leptin polymorphism Q223R in ALA patients showing the significant risk by Q223R mutation. Large population based genotyping studies are needed in Indian population to clarify the susceptibility towards *E. histolytica* infection.

Identification and characterisation of ribosomal DNA (rDNA) transcription factors of *Entamoeba histolytica*

Ankita Srivastava , Gagan Deep Jhingan and Sudha Bhattacharya

School of Environmental Sciences, JNU, New Delhi, India

Initiation of rDNA transcription requires the assembly of a specific multi-protein complex at rDNA promoter containing the RNA Polymerase-I (Pol-I) with many auxiliary factors. RNA Pol-I forms transcriptionally active enzyme, in association with a factor known as Rrn3P in yeast and Transcription Initiation Factor IA (TIFIA, Rrn3P homologue) in mammals. TIF-IA interacts with RPA43, a unique subunit of Pol I, and with two Pol I-specific TAF (TATA binding protein-Associated Factors), thereby serving as a bridge between Pol I and the pre-initiation complex at the rDNA promoter. TIFIA is phosphorylated at multiple sites, and signals in response to that affect cell proliferation and metabolism. In *E. histolytica*, the rRNA genes are present exclusively on 24.5kb circular extra chromosomal plasmid named as EhR1. EhR1 contains two rDNA repeats (I & II), which are arranged palindromically and separated by upstream and downstream spacers. The protein factors involved in regulation of rDNA transcription in *E. histolytica* are not known. We have identified the putative EhTIFIA by sequence analysis and have cloned and expressed the gene in *E. coli*. We have done immunolocalization of the putative EhTIFIA and shown that it is phosphorylated in presence of nuclear extract. Further we are trying to characterize EhTIFIA in relation to RNA Polymerase-I transcription regulation.

Lipids induce expression of serum responsive transmembrane kinase EhTMKB1-9 in early branching eukaryote *Entamoeba histolytica*

Arpita Saha¹, Shiteshu Shrimal^{#1}, Sudha Bhattacharya², Alok Bhattacharya^{1*}

¹ School of Life Sciences, Jawaharlal Nehru University, New Delhi, India,

² School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India,

current address : Umass Medical School , Worcester, Massachusetts, 01605.

The protist parasite *Entamoeba histolytica* is considered to be one of the early branching eukaryotes and is the etiologic agent of amoebiasis, a major cause of morbidity and mortality in developing countries. In our effort to understand the mechanisms of initiation of proliferation we have previously identified a trans membrane kinase EhTMKB1-9 whose expression was found to be regulated by serum using serum replenishment to starved cells as a system. In this study, we show that lipids bound to bovine serum albumin (BSA) are the signal molecules that trigger EhTMKB1-9 expression which was measured by northern analysis and luciferase based EhTMKB1-9 promoter assay. Initial experiments showed that commercial BSA could replace serum during replenishment of starved cells in stimulating expression of EhTMKB1-9. This activation was specific as other proteins , such as mucin did not have any effect and the level of stimulation and time kinetics matched that observed after serum replenishment. PI3Kinase pathway appeared to play an important role in this activation as wortmanin was found to inhibit this activation. Fat free BSA could not stimulate EhTMKB1-9 expression. However, when lipids extracted from BSA were mixed with fat free BSA, the reconstituted mixture was able to stimulate the expression. Moreover, unsaturated fatty acids oleic and linoleic acids also could stimulate expression of EhTMKB1-9 when mixed along with fat free BSA .These results suggest that lipid molecules are ligand(s) for initiation of a signaling system that stimulates EhTMKB1-9 expression. It is one of the few examples of a signaling system that is initiated by lipids.

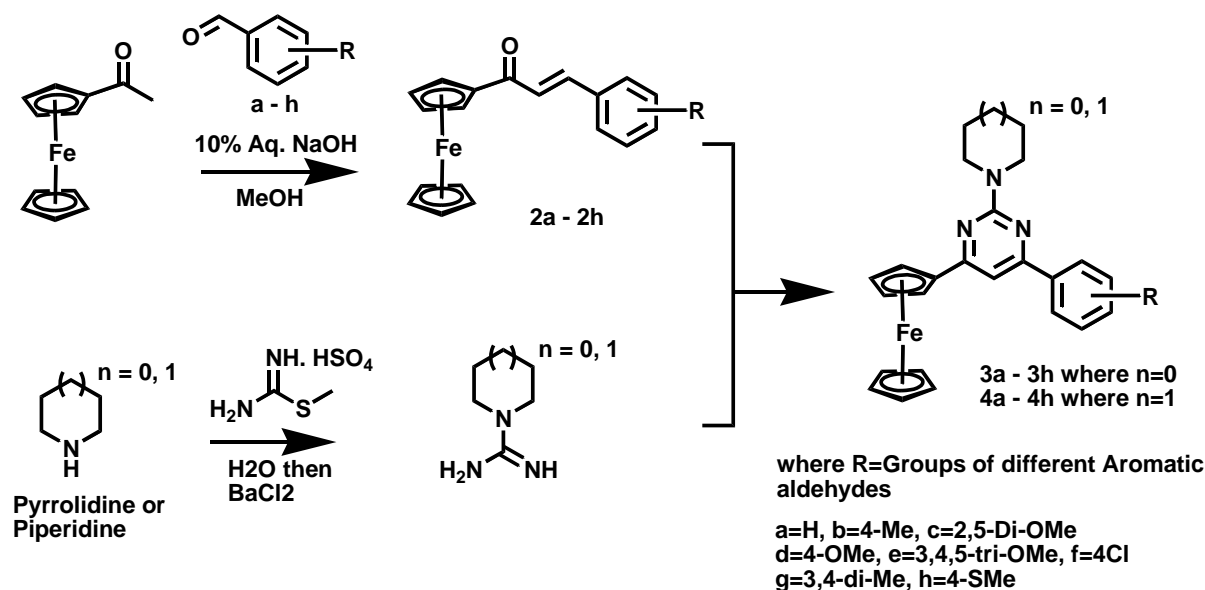
Novel 6-Ferrocenyl-4-aryl-2-substituted Pyrimidine Derivatives as Potent Inhibitors of Protozoan Parasite *Entamoeba histolytica*

Attar Salahuddin^a, Humaira Parveen^a and Amir Azam^a

^a Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India.

A novel series of 6-ferrocenyl-4-aryl-2-substituted pyrimidines were synthesized and evaluated for in vitro antiamoebic activity against HM1: IMSS strain of *Entamoeba histolytica*. Out of 16 compounds 10 compounds have shown IC₅₀ values in the range of 0.41-1.73 μ M and 1.80 μ M. Pyrimidine derivatives having thiomethyl group, chloro group and mono-, di-, and tri-methoxy substitution, exhibited higher antiamoebic activity than the reference drug metronidazole (IC₅₀ =1.80 μ M). The toxicological studies of these compounds on human kidney epithelial cell line showed that all compounds were nontoxic. 4-(4-Chlorophenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4f**) was found most active (IC₅₀ =0.41 μ M) and least toxic among all the compounds.

Keywords: Ferrocenyl Chalcones; Pyrimidine derivatives; Antiamoebic activity, MTT- Assay



Evolutionary divergence and tRNA repeat polymorphism in *Entamoeba histolytica* – Are they correlated?

Avik K Mukherjee¹, Koushik Das¹, Tomoyoshi Nozaki² and Sandipan Ganguly¹

1. National Institute of Cholera and Enteric Diseases, Kolkata, India
2. National Institute of Infectious Diseases, Tokyo, Japan

Amoebiasis, caused by microaerophilic intestinal protozoan parasite *Entamoeba histolytica* is one of the leading causes of morbidity and mortality among all parasitic diseases. It is characterized by symptoms of acute enteric diarrhoea. But in most of the cases the infection remains asymptomatic and very rarely the infection spread to extra intestinal organs like- liver, lungs etc. It is still a paradox that why a single organism shows such a diverse outcome. Genetic polymorphism is a strong hypothesis for this mystery. It is always important to identify the major genotypes in an area before starting an epidemiological study. In this study our attempt was to find out the degree of polymorphism among the local isolates based on selective genetic markers. Detailed sequence analysis of the tRNA linked repeat regions has helped us to find out the presence of unique repeat patterns. The study has also focused on the emergence of new genotypes from the ancestral one due to mere mutation or insertion or deletion. Overall we could establish an evolutionary divergence pathway within the *E. histolytica* species complex.

Leptin signaling protects against *Entamoeba histolytica* infection

Caitlin Naylor¹, Xiaoti Guo^{1,4}, PriyaDuggal², Chelsea Marie^{1,5}, Rashidul Haque³, William Petri¹

¹Department of Medicine, Division of Infectious Diseases and International Health, University of Virginia School of Medicine, Charlottesville, Virginia USA

²Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

³International Center for Diarrheal Disease Research, Dhaka, Bangladesh

⁴present address: Vaccine Research Center, NIAID, National Institutes of Health, Bethesda, Maryland, USA

⁵present address: Department of Biology, Drew University, Madison, New Jersey, USA

Malnutrition increases susceptibility to *Entamoeba histolytica* infection, and suppresses leptin levels. Leptin and leptin receptor signaling has been found to be critical in the host response to *E. histolytica* infection and pathogenesis of the infection. Mice genetically deficient in leptin (ob/ob) or leptin receptor (db/db) were highly susceptible to severe disease. Bone marrow chimeras demonstrated that leptin receptor on hematopoietic cells was not the source of protection. A directed knockout of leptin receptor in the intestinal epithelial cells (IECs) revealed that the critical area of expression and signaling was the IECs. Mutation of tyrosine 1138 in the signaling portion of the leptin receptor abrogated protection in HEK cells, resulting in increased caspase-3 activation. Additionally, children with a glutamine to arginine mutation at position 223 in the extracellular domain of the receptor are significantly more likely to be infected with *E. histolytica*. Mice with the same mutation exhibit a similar phenotype, as well as increased intestinal destruction. When expressed in HEK cells, Q223R receptor led to increased apoptosis and less STAT3 signaling in the presence of leptin. This mutation is believed to alter signaling within the intestinal cell, leading to either direct consequences on the barrier function of the epithelial layer, or an altered ability to effect downstream events. We plan to test the impact of Q223R mutation on IEC signaling and resistance to amebic killing directly on IECs purified from the intestine of mice. Leptin acting indirectly via IEC-recruitment of immune cells will be investigated by characterizing the infiltrating immune populations after an infection with *E. histolytica*. It is hypothesized that mice carrying the Q allele will display decreased apoptosis of the IECs, and/or recruit innate immune cells such as neutrophils more effectively, thus clearing the infection more rapidly. In conclusion, leptin signaling is important for mucosal defense from amebiasis, with polymorphisms within the receptor leading to variable susceptibility to infection.

***Entamoeba bengali*: Identifying a Novel Species of *Entamoeba* in Mirpur, Bangladesh through small subunit rRNA gene sequencing.**

Tricia L. Royer^a, Carol Gilchrist^a, MamunKabir^b, Katherine S. Raleston^a, RashidulHaque^b, C. Graham Clark^c and William A. Petri, Jr.^a

University of Virginia School of Medicine, Charlottesville, Virginia, USA^a, International Centre for Diarrheal Disease Research, Dhaka, Bangladesh^b, London School of Hygiene and Tropical Medicine, London, England^c

The sequences of the small subunit rRNA gene (SSU rDNA) are also now available for several species of morphologically indistinguishable *Entamoeba*. To identify the *Entamoeba* parasites of humans in an endemic area stool samples were collected from a cohort of children from an urban slum in Dhaka, Bangladesh. DNA was extracted from samples positive for *Entamoeba* species by either microscopy or culture. *Entamoeba* parasites that commonly occur in this population (*E. histolytica*, *E. dispar* and *E. moshkovskii*) were identified using specific assays. In samples negative for the ordinary parasites the ENTAGEN-F and ENTAGEN-R primers which exhibit a broad specificity for the SSU rDNA sequences of *Entamoeba* were used to amplify a 459 bp DNA fragment. Upon sequencing two isolates of a novel *Entamoeba* species were identified. This species was named *E. bengali* in recognition of the support of the Bangladesh community for this research. This study confirms that molecular PCR tools are capable of definitive species identification and establishment of taxonomic relationships within this genus. This technique is an effective way to examine the diversity of *Entamoeba* parasitic species in human stool specimens.

Molecular epidemiology of *Entamoeba histolytica* based on the phylogeny of 6 tRNA-linked short tandem repeat loci

Wei-Cheng Yang¹, Jung-Sheng Chen², Shih-Fen Hsu², Chien-Shun Chiou², Wei-Chen Lin² and Dar-Der Ji^{1,2}

¹Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, ²Research and Diagnostic Center, Centers of Disease Control, Taipei, Taiwan

Amoebiasis caused by *Entamoeba histolytica* is still an important parasitic disease in the world and causes about 100 thousands death each year. In Taiwan, the cases of amoebiasis are increasing in foreign worker and HIV/MSM groups, but sporadically outbreak in institutionalized patient group in recent years. Prevalence of amoebiasis is higher in younger Taiwanese population (medium age 34). Study of the strain variations could reveal their genetic connection and transmission pattern among humans. We therefore developed a phylogenetic assay of *E. histolytica* strains based on six tRNA-linked STR loci. A total of 122 *E. histolytica* strains from Taiwanese patients and foreign workers were analyzed. The information of sequence and STR variation were used for the phylogenetic analysis by UPGMA and minimum spanning methods. Two different genotypes were found in two different clustered HIV/MSM groups. Ninety percentages of cases of foreign worker came from Indonesians and were asymptomatic. Strains from same institution might be derived from a common ancestor. This study may provide a new understanding in the amebic genotypes, geographic origins and clinical symptoms among high risk groups, which may be used in further disease investigation and control.

Computational prediction and validation of C/D and H/ACA box snoRNAs and U3 snoRNA of *Entamoeba histolytica*

Devinder Kaur¹, Rahul Sharma¹, Vandana Kumari¹, Abhishek Kumar Gupta¹, Alok Bhattacharya², Sudha Bhattacharya¹

¹School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India, ² School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

Small nucleolar RNAs (snoRNA) play a vital role in biogenesis and processing of pre ribosomal RNA by guiding the chemical modifications required for generation of mature ribosomal RNA or guiding the site specific cleavage of rRNA during pre-rRNA processing. There are two main classes of snoRNA: C/D box and H/ACA box snoRNAs which are associated with 2'-O-ribose methylation and pseudouridylation of pre-rRNA molecules respectively. In this study we have computationally predicted 110 snoRNAs in *Entamoeba histolytica* which includes 37 C/D guide snoRNAs, 7 C/D orphan snoRNAs, 51 H/ACA guide snoRNAs and 15 H/ACA orphan snoRNAs using the tools Snoscan and snoSeeker. We looked for the genomic localization of these snoRNAs and found that 69% snoRNAs are intergenic, 20% are nested in ORF of protein coding genes, and 7.2% are predicted to be present in UTR region while the rest (2.7 %) are variously localized to intron of ribosomal protein S4, ORF2 of LINE1 and 5' end terminus of LINE2. We have experimentally validated 24 snoRNAs, representing all categories on the basis of function and genomic localization using Reverse Transcriptase-PCR and northern blotting. For C/D box snoRNAs, the sizes of observed snoRNAs were consistent with their predicted sizes (60-120 nt). However H/ACA snoRNAs showed deviation from predicted sizes (120 – 160 nt). The observed sizes (60-120 nt) of H/ACA snoRNA shows the possibility that they are present as both single and double hairpin RNA; however this needs further confirmation. We have also shown the presence of five copies of U3 snoRNA localized to intergenic regions of *E. histolytica*, using BLASTn analysis, and validated by RT-PCR and northern blotting.

Identification and analysis of virulence-associated genes in Japanese clinical isolates

Emi Sato¹, Kumiko Nakada-Tsukui¹, Aleya Escueta¹, Makoto Kuroda², Tsuyoshi Sekizuka², Tomoyoshi Nozaki^{1,3}

¹ Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan;

²Laboratory of Bacterial Genomics, Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan; ³Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan

It is known that disease outcomes such as colitis, dysentery, and liver abscess occurs only 5-10 % of people infected with *E. histolytica*. Although it is assumed that host and parasite genetic background likely play important roles, the factors that determine the outcome of infection are poorly understood.

In this study, we performed comparative genomic analysis of pathogenic and non-pathogenic Japanese clinical isolates to identify differentially present genes and genomic regions, which may be associated with virulence. Three clinical isolates each from diarrhea, liver abscess, and asymptomatic patients were subjected to whole genome sequencing by Illumina next generation sequencer. Sequence reads were analyzed by mapping to the HM-1 reference genome or MegaBlast search to the reference after assembled to generate the contig of each strain. These analyses suggested that a non-pathogenic strain derived from asymptomatic case apparently lost a gene of AIG1 family protein. The unique deletion of the gene was further confirmed by PCR. Furthermore, it also demonstrated by PCR analysis that at least two additional genes which are located upstream and downstream of the gene were also missing. Interestingly, these two additional genes also encode AIG1 family proteins. These data suggest the potential importance of this genomic region for *E. histolytica* virulence.

The AIG1 family protein was originally found to be involved in the regulation of immune response in *Arabidopsis thaliana* and also known to regulate apoptosis in mammals. However, a specific role of the gene(s) remains largely unknown in *E. histolytica*. To get insight into the function of AIG1 family proteins in *E. histolytica*, we are currently generating overexpressing and gene silenced strains, which we will present and discuss.

HLA-DRB1 and HLA-DQB1 alleles in patients with Amoebic Liver Abscess caused By *Entamoeba histolytica* in The Mexican Population

Eric G. Hernández Hernández¹, Oswaldo Partida-Rodríguez¹, Miriam Nieves-Ramírez¹, Olivia Valenzuela Antelo², Ulises Magaña Nuñez², Patricia Morán Silva¹, Liliana Rojas Velazquez¹, Enrique González Rivas¹, Alicia Valadez Salazar¹, Rene Cerritos Flores¹, Valeria Zermeño León¹, Mónica Escamilla Tilch³, Julio Granados Arriola³, Cecilia Ximénez García¹.

¹Department of Experimental Medicine, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Mexico, Federal District.

²Department of Chemistry and Biological Sciences, University of Sonora (UNISON), Hermosillo, Sonora, Mexico.

³National Institute of Sciences and Nutrition Salvador Zubiran, Mexico, Federal District.

The intestinal protozoan *Entamoeba histolytica* (*E. histolytica*) infects 50 million people worldwide. About 40,000 to 100,000 die annually by amebiasis complications that include dysentery and extra intestinal diseases as amoebic liver abscess. In Mexico the prevalence of *E. histolytica* infection is 13.8%, but the amoebic liver abscess is particularly high in the north of the country (Sonora with 12.57/100,000, incidence rate in 2005) compared with the national average (3.66/100,000), suggesting a possible genetic influence on susceptibility. The Mexican population shows great genetic heterogeneity, so the aim of this work was to determine HLA class II resistance or susceptibility alleles to amoebic liver abscess in two Mexican populations from central (Federal District) and northern (Sonora) area of the country. We studied the allele frequencies of HLA-DRB1 and HLA-DQB1 by PCR-SSO in patients with amoebic liver abscess (N= 64) and healthy blood donors (N= 99). Infection was determined by detection of IgG-specific antibodies to *E. histolytica* by ELISA. Genomic DNA was extracted using a salting-out microtechnique. Interestingly, we found an increased frequency of HLA-DRB1*01 in patients with amoebic liver abscess (0.150) when compared with controls (0.050) of the state of Sonora, while in the Federal District the HLA-DRB1*04 were more frequent in amoebic liver abscess patients (0.323) when compared against the control group (0.237). On the other hand the allele HLA-DQB1*030101 were less frequent in patients from both localities (0.166 in Sonora and 0.220 in the Federal District) compared to their respective control subjects (0.229 and 0.315 respectively). Although the number of samples continues to increase, our preliminary results suggest a possible risk association of HLA-DRB1*01 and HLA-DRB1*04 alleles with amoebic liver abscess caused by *E. histolytica* infection, whereas DQB1*030101 allele appears to protect against disease development in Mexican population.

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Molecular and morphological identification of pathogenic free-living amoebae in superficial water in Shiraz, Iran

Hatam G.R., Mohammadi-ghalehbin B., Motazedian M.H., Mohammadpour I., ghobakhloo N.

Center for Basic Researches in Infectious Diseases, Shiraz University of Medical Sciences, Shiraz, Iran

Introduction: Among the many genera of free-living amoeba that exist in the nature, members of only four genera (*Negleria*, *Acanthamoeba*, *Balamuthia*, *Sappinia*) have an association with human infection. These genera are known as causative agents of many clinical manifestations such as encephalitis and keratitis. Infective forms of these pathogenic organisms distribute in wide geographical regions of the world and consider in water, soil and air. There are only a few studies have done about these protozoa in Iran. **Materials and methods:** 30 samples were collected from superficial water of shiraz in summer 1388. The samples were filtered and their sediments were cultured on NNE medium and were incubated at three different temperatures; 22 °C, 37 °C, 44 °C. The media were inspected with Invert microscopy and amoebae were identified by phase-contrast microscopy and were evaluated by light microscopy after trichrom staining. Polymerase chain reaction (PCR) was performed for molecular detection of amoebae. **Results:** Among 30 samples, 29 out of them were identified as *Acanthamoeba* by microscopy and confirmed by PCR. The growth rate of amoeba in 22 °C were more than 37 °C. Eight of 30 samples grew at 44 °C, but flagellate forming test of them were negative and showed any band with *N. fowleri* specific primers. Two of samples were identified morphologically as *Balamuthia* and *Sappinia*. **Conclusion:** Due to the wide distribution of free living amoeba in superficial waters in Shiraz, further investigations about various aspects of this important opportunistic protozoa especially for establishment of appropriate prevention tools is recommended.

Key words: Free –living amoeba, *Acanthamoeba*, PCR, Shiraz

Biochemical and functional analysis of EhMAPK along with proteogenomic analysis during various growth conditions in *E. histolytica*

Gagan Deep Jhingan¹, Sudha Bhattacharya², Alok Bhattacharya² & Vinay K. Nandicoori¹

Jawaharlal Nehru University,²New Delhi 110067,India,National institute of Immunology¹, Aruna Asaf Ali Marg, New Delhi 110067, India

MAP kinases respond to extracellular stimuli (mitogens) and regulate various cellular activities including growth and phagocytosis via an evolutionary conserved MAP Kinase signaling cascade. Unlike mammals where three MAP kinases (p38, ERK and JNK) are well studied, *Entamoeba* contains only one MAP kinase homolog (EhMAPK) which is similar to mammalian ERK2. We have demonstrated that differential phosphorylation pattern exists during autophosphorylation and transphosphorylation of EhMAPK kinase. It was observed that autophosphorylation takes place at tyrosine residue of the activation loop while multiple serine residues were responsible for the trans-phosphorylation in the presence of amoebic cell extracts. The nucleo-cytoplasmic shuttling behavior of EhMAPK was found to be sensitive to cellular growth and stress conditions. We further utilized proteogenomic analysis for identification of rare post-translational modifications for which the high-throughput techniques are not available. Drawing parallels from gene microarray platforms, we also used mass spectrometry based protein expression data to analyze the conserved and differentially expressed signalling pathways during various growth conditions.

Comparative analysis of Ubiquitin Proteasome Pathway in Entamoeba species

Gaurav Sharma, Shweta Arya, Richa Gupta, and Swati Tiwari

School of Biotechnology, Jawaharlal Nehru University, New Delhi, India

Ubiquitin Proteasomal pathway (UPP), a key regulatory process to degrade mutated or misfolded proteins comprises of three enzyme classes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). The complexity in life cycle and multiple hosts of protozoan parasites requires a coordinated change in the transcriptional and proteomic profile in protozoa. Our hypothesis is that UPP may be playing an important role in these processes.

We have used bioinformatics tools to identify and compare UPP components in three members of Entamoeba species namely *E. histolytica*, *E. dispar* and *E. invadens*. Some classical families of E2s that function in mitotic substrate modifications in other eukaryotes are absent in Entamoeba. Majority of identified E3s could not be functionally annotated based on similarity to known E3s and presence of some unique E3s suggested parasite specific functions. Domain architecture of RING finger E3s suggests modulation of a wide variety of functions by these enzymes. Most of the E2 and E3 genes were present in syntenic positions in *E. histolytica* and *E. dispar*. However, 20% RING finger E3s were closely associated with transposable elements (TEs) in *E. histolytica* compared to only 9% in *E. dispar*. The clusters of TEs were more complex in *E. histolytica* than those present in *E. dispar*. E2 genes, however, were not closely associated with TEs. GEO datasets showed differential expression of an ubiquitin-like modifier Nedd8, and a number of E2 and E3 enzymes in *E. histolytica*. Orthologs of these genes are conserved in *E. invadens*, Semi-quantitative PCR of some of these genes in *E. invadens* showed differential expression in the two developmental stages.

These studies provide a useful resource to study this important pathway in Entamoeba. We are currently trying to study the role of UPP in the developmental changes in *E. invadens*.

Metabolomics of parasite differentiation: metabolomic profiling of the protozoan parasite *Entamoeba* revealed activation of unpredicted pathways during encystation

Ghulam Jeelani^{a,b}, Dan Sato^c, Afzal Husain^a, Aleyla Escueta-de Cadiz^a, Makoto Suematsu^b, Tomoyoshi Soga^c and Tomoyoshi Nozaki^a

^aDepartment of Parasitology, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan

^bCenter for Integrated Medical Research, School of Medicine, Keio University, Shinjuku, Tokyo 160-8582, Japan

^cInstitute for Advance Biosciences, Center for Integrated Medical Research, School of Medicine, Keio University, Tsuruoka, Yamagata, 997-0052, Japan

Several extracellular parasitic protozoa present two morphologically distinct stages in their life cycle: the labile trophozoite, or motile form, which inhabits the host, and the resistant cyst, protected by a cell wall that allows survival under the adverse external environment. Cysts and their formation are integral to the transmission of the disease. Thus, attacking the formation of cysts may represent a potential useful approach for chemotherapeutic against the protozoal diseases. Therefore, it is important to know the biochemical and metabolic changes which occur when the parasite differentiate from trophozoites to cysts form. Invitro encystation has been investigated using *Entamoeba invadens*, the reptilian sibling of *Entamoeba histolytica*. Using capillary electrophoresis-tandem mass spectrometry based metabolites profiling methods; we have determined the intracellular concentrations of many metabolites during encystation. Our data revealed that the concentrations of the majority of metabolites drastically decreased during encystation, whereas the concentrations of the metabolites involved in cyst wall biosynthesis increased. We also found remarkable changes in biogenic amine metabolites such as cadaverine, isobutylamine and isoamylamine, which increased during the early period of encystation, when the trophozoites form large multicellular aggregates (precyst), and then decreased when the precyst differentiates to the cyst form suggest that these metabolites play an important role in inducing the encystation process. We have also identified a pathway which is involved in γ -aminobutyric acid (GABA) synthesis which is induced during encystation. Significance of these metabolomic changes in encystation will be discussed.

Phenotypic and transcriptional profiling in *Entamoeba histolytica* reveal costs to fitness and adaptive responses associated with metronidazole resistance

Gil M. Penuliar^{1,2,3}, Kumiko Nakada-Tsukui¹ and Tomoyoshi Nozaki^{1,4}

¹Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan,

²Department of Parasitology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi 371-8511, Japan,

³ Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City 1101, Philippines,

⁴ Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Antimicrobial chemotherapy is critical in the fight against infectious diseases caused by *Entamoeba histolytica*. Among the drugs available for the treatment of amoebic dysentery and amoebic liver abscess, metronidazole (MTZ) is considered the drug of choice. Treatment failures, however, have been documented. Recently, in vitro studies on MTZ resistance have described the potential mechanisms involved, but costs to fitness and adaptive responses associated with resistance have not been investigated. In this study we generated an HM-1 derived isogenic strain resistant to 12 μ M MTZ (MTZR). We examined its phenotypic and transcriptional profile against the parental strain to determine the consequences and mRNA level changes associated to MTZ resistance. Our results indicated an increase in size and granularity, and decreased rates in cell division, adhesion, phagocytosis, cytopathogenicity, and glucose consumption. Transcriptional profiling also revealed 142 genes that were differentially expressed in MTZR. In contrast to MTZ resistance in other protozoan parasites, MTZR did not down-regulate pyruvate: ferredoxin oxidoreductase, but showed increased expression in a hypothetical protein (HP1) with some similarity to a zinc finger protein, several iron-sulfur flavoproteins, and downregulation of leucine rich proteins. Overexpression of HP1 did not confer MTZR level of resistance, but provided slight advantage in cell survival when exposed to MTZ for 48h. Fisher's exact test showed 24 significantly enriched GO terms in MTZR, which could be classified under nucleotide binding, metabolism, oxidative stress response, and signal transduction. A three-way comparison of differentially expressed genes against MTZR(-) and HM-1(+) also showed that 88 genes were distinctly modulated in MTZR. Overall, our findings suggest that decrease in parasite virulence and specific transcriptional changes are associated with MTZ resistance.

Expression of peroxiredoxin by RT-PCR and its immunolocalization in standard laboratory strain and patient derived strains of *Entamoeba*.

Iyer Lakshmi Rani¹, Singh Nishant², Haque Rashidul³, Paul Jaishree¹ and Bhattacharya Sudha².

1. School of Life Sciences, Jawaharlal Nehru University, New Delhi 110070.
2. School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110070.
3. Parasitology Laboratory, I.C.D.D.R, Dhaka, Bangladesh.

Entamoeba histolytica trophozoites reside and multiply in the human gut. During tissue invasion, however, the parasite is exposed to elevated levels of reactive oxygen and nitrogen species. Peroxiredoxins are a family of antioxidants that protect the cell from metabolically produced reactive oxygen species. In this study the expression levels of peroxiredoxins were studied by RT-PCR in clinical isolates of *Entamoeba histolytica*, MS-96 (Bangladesh) and patient isolated strains 654 and 812 from India and compared with standard strain HM1:IMSS. The peroxiredoxin expression levels were similar in the axenised patient strain MS-96 to HM1:IMSS, while the patient strains 654 and 812 grown in xenic culture medium showed lower expression of peroxiredoxin.

Peroxiredoxin localization was done in the standard strain HM1: IMSS and in clinical isolates of *Entamoeba histolytica* and *Entamoeba dispar* using a monoclonal antibody. Our study revealed that peroxiredoxins are localized both to the nucleus and to the cell membrane in HM1: IMSS. In an axenised patient strain, MS96, immunolocalization of peroxiredoxin occurred in the nucleus and in the cytoplasm; however during pseudopodia formation peroxiredoxin localization in the cell membrane was also noted. Patient isolates 812 and 654 in xenic culture also showed similar membrane localization.

Entamoeba dispar isolates 718 and 724 also showed nuclear and cytoplasmic localization of peroxiredoxin.

During both serum starvation and metronidazole stress, the strain HM1:IMSS showed peroxiredoxin localization in membrane, while in cells which were adapted to growth in metronidazole, the peroxiredoxin localized to the nucleus and cell membrane. The different localization of peroxiredoxin in the *Entamoeba* strains is related to its role in enabling the organism adapts to the different environments.

DNA Based Molecular and *In-Silico* Tools in Diagnosis of Causative Agents of Amphistomiasis

J.A.Shylla¹, S.Ghatani¹, V.Tandon^{1,*}, A.Chatterjee²

¹ Department of Zoology, North-Eastern Hill University, Shillong 793022, Meghalaya, India

² Department of Biotechnology, North-Eastern Hill University, Shillong 793022, Meghalaya, India

Amphistomid (Platyhelminthes: Trematoda: Digenea) flukes are the main causative agents of the debilitating disease called Amphistomiasis. It poses a major obstacle to livestock production in ruminants worldwide, especially in young animals where it causes high morbidity and mortality. These flukes are extremely difficult to identify possibly due to the great morphological similarities exhibited by these trematodes. Molecular characterization of these flukes allows for a quick and accurate identification of genetically distinct but morphologically similar species. DNA based molecular tools have proven to be invaluable in identifying digenean species and to recover their phylogenetic relationships. Multiple genetic markers viz., second internal transcribed spacer (ITS2), cytochrome oxidase1 (CO1) and large ribosomal subunit DNA (LSR) were amplified and the sequences were analysed to ascertain the identification of these ambiguous flukes. Molecular morphometrics using the secondary structure was used in order to offer an additional source of information for species identification and phylogenetic tree reconstruction. Restriction fragment length polymorphisms in polymerase chain reaction amplified fragments (PCR-RFLP) of ITS2 were also used to further differentiate these species. The study also established molecular markers to differentiate between different species of paramphistomes and thus assist in authentic species identification supplementing the morphological criteria.

Regulation of ribosomal protein genes in *E. histolytica* under serum stress condition

Jamaluddin Ahamad and Sudha Bhattacharya

School of Environmental Sciences, JNU, New Delhi, India

Ribosome synthesis is a fundamental and complex process that accounts for a large segment of total energy consumption by the dividing cells. In eukaryotes, this process occurs sequentially in the nucleolus, nucleoplasm and the cytoplasm. It involves the transcription and processing of pre-ribosomal RNAs, their proper folding and assembly with ribosomal proteins and transport of the pre-ribosomal particles to the cytoplasm for the final maturation. The ribosome production involves the coordinated synthesis of four rRNAs, about 80 ribosomal proteins, more than 150 other proteins, and about 70 snoRNAs. We have found that under growth stress conditions the pre-rRNA accumulates in *E. histolytica*, and we are interested to explore whether the ribosomal protein expression is co-ordinately regulated along with pre-rRNA. We have selected two ribosomal protein genes (RPS19 and RPL30) for our analysis. We are looking at the levels of their mRNAs during the serum starvation, by northern analysis. We have also cloned the upstream regulatory regions of these genes next to luciferase reporter gene and obtained stable transfectants. The results of these analyses will be presented.

EhCaBP6 is an essential component of mitotic apparatus in *Entamoeba histolytica*.

Jaspreet Singh Grewal¹ and Anuradha Lohia¹

¹ Department of Biochemistry, Bose Institute, Kolkata, India

The genome of *Entamoeba histolytica* encodes several calcium binding proteins and those characterized so far have been shown to participate in the processes of phagocytosis and endocytosis. The focus of our study is to investigate the role of a novel calcium binding protein, EhCaBP6, with respect to the cell cycle of the parasite. EhCaBP6 is the member of the EF-hand family of calcium binding proteins. Immuno-localization studies reveal that EhCaBP6 is a nucleo-cytoplasmic protein and it maintains nuclear localization throughout the nuclear cycle of the trophozoites. The protein concentrates at the interface of microtubules and chromosomes in mitotic cells. Using the yeast two hybrid screen we identified two novel interactors that bind to EhCaBP6. These were a Zn finger protein and the Ribosomal protein P2. Our studies that EhCaBP6 is an essential component of the mitotic assembly.

Expression and functional analysis of LINE1-encoded ORF1 of *Entamoeba histolytica*

Jitender Kumar¹, Alok Bhattacharya² and Sudha Bhattacharya¹

¹School of Environmental Sciences, JNU, New Delhi, India , ²School of Life Sciences, JNU, New Delhi, India

Retrotransposons can be classified into two groups: Long Terminal Repeat (LTR) retrotransposons and non-LTR retrotransposons. *Entamoeba histolytica* contains retrotransposons belonging to non-LTR class, which is further divided into two categories long interspersed elements (LINEs) and short interspersed elements (SINEs). *E. histolytica* LINE contains two ORFs, ORF1 encodes a protein that is supposed to be a nucleic acid binding protein, and ORF2 contains a centrally located reverse transcriptase domain and an endonuclease domain. LINEs are further divided into three classes of LINEs: LINE1, LINE2, and LINE3. Till now there is no evidence of full length active copy of *E. histolytica* LINEs.

Though most copies of *E. histolytica* LINEs have accumulated multiple mutations, we have found the expression of the ORF1 protein of *E. histolytica* LINE1 in vivo by western blot analysis. We have studied the expression of ORF1 mRNA in *E. histolytica*. In which we found that the multiple copies of the ORF1 express in vivo. We find that the expression of ORF1 changes under the heat stress but not in other stress like oxygen and serum starvation. By immunolocalization we find that in normal cells ORF1 resides in both nucleus and cytoplasm. However under heat stress it disappears from cytoplasm. Our preliminary data shows that ORF1 is a nucleic acid binding protein. We plan to detect the intracellular interacting partners of this protein to understand its possible physiological role apart from that in retrotransposition.

Identification of *Entamoeba histolytica* DNases

Julia Matt and Michael Duchêne

Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria.

Metronidazole is the gold standard drug to treat invasive *Entamoeba histolytica* infections. In metronidazole-treated amoebae DNA damage can be observed in vitro, but it is not known whether this DNA damage is the cause or the consequence of the breakdown of cellular processes. Moreover, it is not known if the DNA is damaged chemically by metronidazole metabolites, such as the nitroradical anion, or enzymatically by DNases from the parasite. For a better understanding of this question, different attempts were made to identify nuclease activities. In the *E. histolytica* genome database 58 genes coding for nucleases (excluding RNases) were found. We found eight gene products annotated as endonuclease V, plus many further variant genes, but no classical DNases I or II, caspase-dependent DNase, or endonuclease G.

Incubation of *E. histolytica* lysate with plasmid pUC19 DNA resulted in endonucleolytic digestion, stimulated by Mg^{++} ions, as shown by agarose gel electrophoresis or UV photometry. The activity found in the lysate was different from the additional DNase activity in the bovine serum added to the culture medium. To enrich the amoebic DNase(s), the eluate fractions of anion exchange chromatography (ÄKTA Purifier) were analyzed in a fluorescence based DNase detection assay. For this purpose a stem-loop DNA beacon probe labeled with the fluorophore FAM and the quencher BHQ-1 was constructed. Two-dimensional gel electrophoresis was performed with fractions of high DNase activity with the aim of mass spectrometric identification, but so far none of the analyzed spots were found to be a DNase. The ultimate aim of the work is to understand the function of the DNase or DNases in *E. histolytica* and its or their possible role in the mechanism of metronidazole action.

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Amoebic liver abscess: An emerging threat in northern Sri Lanka

Janani T¹, Pushpana P¹, Surenthirakumaran R¹, Kumanan T² and Kannathasan S³

¹Department of Community Medicine, Faculty of Medicine, University of Jaffna, Sri Lanka

²Department of Medicine, Faculty of Medicine, University of Jaffna, Sri Lanka

³ Division of Parasitology, Department of Pathology, Faculty of Medicine, University of Jaffna, Sri Lanka

During the recent past, many cases of clinically diagnosed Amoebic Liver Abscess (ALA) patients were admitted to the Teaching Hospital Jaffna, Sri Lanka. With this background, a descriptive study, using an interviewer administered questionnaire was carried out to find out the risk factors of ALA among clinically and ultrasonologically confirmed patients with ALA. All the patients (110) admitted during the period from March to August 2011 were included in this study. Among them, 98% were male and half (50%) of them had only primary education. Majority (91%) were from urban area and 32% within the age group of 50-60. Eighty eight respondents (80%) earn low level of income and 72% were manual workers. Most of them (80%) obtained drinking water from unprotected well and 25% practiced open air defecation. Majority (97%) were alcoholic; among them 78% taking alcohol for more than 5 years, mainly “toddy” (69%) and (59%) consumes daily. Most of them (80%) drank “toddy” at “tavern” where 88%, defecated after having “toddy” and among them 85% defecated in the open air. Ninety nine (83%) respondents stated that the utensils used in the “tavern” were not properly cleaned. Few (12%) had previous history of ALA and 17% could recall the past history that they had blood and mucous diarrhea.. Based on chi-square test, sex, occupation, poor economic status, alcoholic habit , drinking “toddy” , open air defecation at “tavern” and improper washing of utensils at “tavern” are the statistically significant (at p value -0.05 level) risk factors of ALA. In conclusion, increasing the hygienic practices such as drinking water from protected wells, defecating in water sealed toilets especially at “tavern” and proper and frequent cleaning of utensils used at “tavern” combine with public awareness programs may reduce the future incidents of ALA in Jaffna.

Identification of ligands and regulatory/accessory proteins associated with cysteine protease binding protein family in *Entamoeba histolytica*

Konomi Marumo^{1,2}, Kumiko Nakada-Tsukui¹, Naoko Watanabe², Tomoyoshi Nozaki^{1,3}

¹Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; ²Department of Molecular Biology, Faculty of Science, Toho University, Chiba, Japan; ³Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan

Lysosomal enzymes play a pivotal role in the pathogenesis of *Entamoeba histolytica*. In vertebrates, only two major hydrolase receptors have been described that mediate the trans-Golgi network to lysosome transport of cargo enzymes by recognizing their phosphorylated carbohydrate epitopes. We recently identified a novel class of lysosome targeting receptor family proteins [cysteine protease binding protein family (CPBF)], which are conserved exclusively in *Entamoeba* species. Each CPBF binds to different cargo molecules: e.g., CPBF1, CPBF6, and CPBF8 bind to EhCP-A5, amylases, β -hexosaminidase and lysozymes, respectively. However, ligands and regulatory/accessory proteins of other CPBF members have not yet been identified.

In this study, we tried to identify the ligands and accessory (associated) proteins of CPBF3, 4, 10, and 11 to understand mechanisms that regulate localization of CPBFs. *Amoeba* transformants that express HA-tagged CPBF3, 4, 10, or 11 were established. Localization of these CPBFs was similar to that of CPBF1, suggesting their involvement in the ER to lysosomal transport. Immunoprecipitation of HA-tagged CPBFs revealed specific interacting proteins associated with CPBF3-11. We will present the result of mass spectrometric analysis, and discuss their potential roles in the regulation of lysosomal trafficking.

***Naegleria fowleri* HSP70 partially involved in cytotoxicity and ROS production in Jurkat T cells via TLR2 and TLR4**

Kyoung-Ju Song, Myeong Heon Shin

Department of Environmental Medical Biology, Institute of Tropical Medicine, College of Medicine Yonsei University, Seoul 120-752, Korea

Naegleria fowleri, a free-living amoebae, is the causative pathogen of primary amoebic meningoencephalitis in humans and experimental mice. Upon host invasion, *N. fowleri* is capable of destroying tissues and host cells through lytic necrosis. However, the signaling mechanism by which *N. fowleri* induces host cell death via necroptosis is unknown. In the present study, we investigated signaling pathway and molecules in *N.fowleri* induced Jurkat T cell death. When Jurkat T cells are incubated with secretory products (NfSP) and trophozoites of *N. fowleri*, the release of extracellular lactate dehydrogenase (LDH) is increased with time and dose dependent manner and amoeba-induced ROS generation as known as important signaling molecules in cell death is significantly increased. Blocking TLR2 or 4 mediated signaling by pretreatment of Jurkat T cells with specific Ab significantly prevented the NfSP and trophozoites induced ROS generation and cell death. Previous studies have described the *N.fowleri* derived heat shock protein 70 (Nf-HSP70) is an important factor in host cell cytotoxicity. Extracellular ROS and cytotoxicity were induced by treatment with recombinant Nf-HSP70 protein and reduced by treatment with Nf-SP (HSP70 synthesis inhibited with pretreatment with KNK437 as HSP70 inhibitor) and blocking with TLR2 and TLR4 antibodies.

These results suggest that *N. fowleri* induce cell death in Jurkat T cells via TLR2 and 4 mediated ROS-dependent signaling, especially Nf-HSP70 protein was partially involved in cytotoxicity and ROS production in Jurkat T cells via TLR2 and TLR4.

Structural characterization of cysteine proteases inhibitors of *Entamoeba histolytica* sheds light into its evolutionary adaptations

Itzel Renteria¹, Samuel Lara¹, Luz Casados¹, Claudia Benitez², Elisa Azuara³, Federico del Rio⁴, Luis Briebe¹

¹ National Laboratory of Genomics-Cinvestav-IPN, Irapuato Mexico

² School of Medicine, National Polytechnic Institute, Mexico City, Mexico

³ Posgrado en Ciencias Genómicas, Autonomous University of Mexico City, Mexico

⁴ Institute of Chemistry, National University of Mexico, Mexico City, Mexico

Entamoeba histolytica contains at least 50 cysteine proteases in its genome and two cysteine protease inhibitors (EhICP1 and EhICP2). Previous biochemical studies had demonstrated that both EhICPs display selectivity for targeted proteases and differential sub-cellular localization. However, the mechanisms by which EhICPs display selectivity and are regulated are unknown. Our biochemical studies of EhICP1 indicate that this inhibitor is prone to form inactive dimers which formation is mediated by disulfide cross-linking. We have constructed a triple EhICP1 mutant that is able to form crystals that diffract at 3.1 Å and the structure of EhICP1 is being tackled by NMR and X-ray crystallography. We have solved the crystal structure of EhICP2 and shown that this inhibitor reduces dramatically the protease activity of papain, demonstrating that indeed this is an “active” protease inhibitor. The crystal structure of this inhibitor demonstrates that EhICP2 has an immunoglobulin fold and that the inhibitory loops are quite flexible.

A biophysical characterization of EhICP1, EhICP2 and chagasin illustrates that the folding pathway of EhICP1 is different than the folding pathway of EhICP2 and chagasin. This study corroborates a phylogenetic relationship in which EhICP2 and chagasin share a closer ancestor. We propose a model in which the inhibitory activities of EhICP1 are modulated by redox potential and demonstrated that the change of the conserved Thr by Ser in an interacting BC loop of EhICP2 does not hamper the inhibition of EhICPs to papain.

Identification and Characterization of Ehak1: a SH3 domain containing alpha kinase in *Entamoeba histolytica*

M. Shahid Mansuri¹, Sudha Bhattacharya², and Alok Bhattacharya^{1,3}.

¹ School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India,

² School of Environment Sciences, Jawaharlal Nehru University, New Delhi-110067, India.

³ School of Computational & Integrative Sciences, Jawaharlal Nehru University, New Delhi-110067, India

Phagocytosis is an essential process for survival and virulence in *Entamoeba histolytica*. However, molecular mechanisms controlling this process are not very well understood. Our group has already shown that calcium binding protein EhCaBP1 is involved in initiation of phagocytosis. One of the putative alpha kinases (Ehak1) was identified as an EhCaBP1 binding protein by affinity chromatography followed by mass spectrometric analysis. Ehak1 belongs to the atypical class of alpha kinase family that phosphorylate substrates with high fraction of alpha helical domains. It has a conserved SH3 and a kinase domains and was expressed and purified as His-tagged recombinant protein. The purified recombinant protein was found to be catalytically active and bound EhCaBP1 in vitro. Biochemical analysis showed that Ehak1 is a Mg²⁺ dependent serine/threonine kinase. It has three auto-phosphorylation sites at Ser⁵⁴, Thr²⁷⁹ and Ser³⁹⁸ as revealed by mass spectrometric analysis and confirmed by mutation study. Our data suggests that phosphorylation is mediated by bimolecular-trans reaction. Functions of all three autophosphorylation sites (S54A, T279A, S398A) were investigated with respect to auto and substrate phosphorylation. Immunofluorescence studies revealed that Ehak1 is a cytosolic protein and was also found in phagocytic cups during erythrophagocytosis. The data presented here suggest that Ehak1 may be involved in the erythrophagocytosis in *E. histolytica*.

Autophosphorylation of Ser428 of EhC2PK plays a critical role in regulating erythrophagocytosis in the parasite *Entamoeba histolytica*

Somlata¹, Mrigya Babuta¹, Sudha Bhattacharya² and Alok Bhattacharya^{1,3}

¹ School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India,

² School of Environment Sciences, Jawaharlal Nehru University, New Delhi-110067, India.

³ School of Computational & Integrative Sciences, Jawaharlal Nehru University, New Delhi-110067, India

Our laboratory is interested in deciphering the mechanisms involved in the initiation of phagocytosis in *Entamoeba histolytica* as it is a crucial activity for the survival and virulence of *E. histolytica*. It was shown earlier that the C2 domain containing protein kinase EhC2PK is involved in the initiation of erythrophagocytosis. Cells expressing kinase dead mutant of EhC2PK showed a reduction in erythrophagocytosis suggesting that the kinase activity is necessary for initiation. Biochemical analysis showed that EhC2PK is an unusual Mn²⁺-dependent serine kinase with one ATP binding site. Mass spectrometric analysis showed presence of predominant single autophosphorylated site at Ser428, which in turn was confirmed by biochemical analysis. The autophosphorylation defective mutants (S428A, KDΔC) displayed loss of auto and substrate phosphorylation activities. Time kinetics of in vitro kinase activity suggested two phases, an initial short slow phase followed by a rapid phase suggesting that autophosphorylation may be controlling kinase activity through an autocatalytic mechanism. These results indicate that enrichment of EhC2PK at the site of phagocytosis enhances the rate of autophosphorylation, thereby regulating the initiation of erythrophagocytosis in *E. histolytica*

Structural and functional features of the lysine and glutamic acid rich protein KERP1, a virulence factor of *Entamoeba histolytica*

Doranda Perdomo^{1,2}, Sylvie Syan^{1,2}, Bruno Baron³, Patrick England³ and Nancy Guillén^{1,2}

¹Institut Pasteur, Cell Biology of Parasitism Unit and ²INSERM U786, ³Institut Pasteur, Plate-Forme de Biophysique des Macromolécules, Paris, France

The lysine and glutamic acid rich protein KERP1 from *Entamoeba histolytica* is involved in pathogenesis (1,2) as it has been demonstrated by using parasites invalidated for kerp1 gene expression. KERP1 has no known homologue when compared to proteomes from any other organism. The bioinformatics analysis of the primary structure from this basic protein revealed that there are no signal peptides or relevant predicted domains that would provide an insight into its function during infection. However, three parts of the KERP1 secondary structure are predicted to form coiled coil regions. These motifs are present in proteins whose functions have an impact in pathogen adherence to host cells and invasion. To determine the structural features of KERP1, we employed biophysical tools such as circular dichroism and analytical centrifugation. The data show that KERP1 is folded as an alpha helical trimer with high stability to increasing temperatures. The purified protein binds to phosphoinositides as we have shown by lipid blotting and liposomes-KERP1 interaction experiments. Immunoprecipitation coupled to mass spectrometry analysis allowed us to identify KERP1's binding partners, some of which are involved in vesicle trafficking. To gain insight into the function of KERP1 we followed two approaches. First, confocal microscopy was used to determine the intracellular localization of KERP1 during the interaction of *E. histolytica* with human intestinal cells. This protein is enriched in vesicles released from the trophozoites and these are deposited on the surface of human cells. Second, we assessed the impact of coiled coil motifs on the protein activity by generating a dominant negative KERP1 parasite line by overexpressing the coiled coil peptide. The transfected cell line has reduced abilities for adhesion to cells. Together, these data suggest KERP1 functions as an adhesive molecule to form an intricate network necessary for the infection process.

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Demonstration of homologous recombination in *Entamoeba histolytica* under normal and stress conditions

Nishant Singh¹, Alok Bhattacharya² and Sudha Bhattacharya¹

¹School of Environmental Sciences, ²School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India.

The genome of *Entamoeba histolytica* shows plasticity in terms of chromosome size polymorphism, uncertain ploidy and many classes of repetitive DNA sequences. Homologous recombination (HR) is one of the mechanisms to generate diversity. It is also essential for genome integrity and for repair of DNA double strand breaks. We have computationally identified the *E. histolytica* homologues of genes involved in meiosis and HR and checked their expression by real time PCR and northern analysis. Most of the genes were up regulated during serum starvation in *E. histolytica* and during encystation in *Entamoeba invadens*. For the first time we have demonstrated HR in *E. histolytica* by transfecting the cells with a construct with inverted repeats. Recombination between the repeats will cause sequence inversion which can be detected by PCR with appropriate primers. Using this read out we found that recombination between inverted repeats is enhanced in different stress conditions like serum starvation, heat shock and oxygen shock. In *E. histolytica*, three meiotic genes and many HR specific genes are expressed in serum starvation, although meiosis has not been observed in *Entamoeba*. Our findings will help in further investigation to understand DNA repair, HR and possibilities of meiosis in *Entamoeba*. Further, by enhancing the rate of HR, it may be possible to achieve gene knock-out in *E. histolytica*.

Structural and functional studies of Coactosin from *Entamoeba histolytica*

Nitesh Kumar, Sankar Maiti, Saima Aslam, Somlata and S. Gourinath

School of Life Sciences, JNU, New Delhi

Entamoeba histolytica causes amoebic dysentery and amoebic liver abscess. Survival and pathogenicity of *E. histolytica* requires dynamic alterations in actin cytoskeleton to adhere to host components, to migrate through tissues and to phagocytise human cells. The ADF/Cofilins are the only known essential family of proteins that induce a twist to the actin filament conferring different properties to the structure. Coactosin, a member of this family interacts with F-actin to cause its depolymerisation thus regulating filament architecture, but little is known about the exact mechanism of its action. *Entamoeba* coactosin (EhCoacto) shares 55% homology to its human counterpart. In this study, EhCoacto was cloned, overexpressed and purified by affinity and size-exclusion chromatography. The purified native protein was crystallized at 289K by hanging drop vapour diffusion method using PEG1500 and diffracted X-rays to 1.8Å resolution. The crystals belonged to C2 space group, but the structure could not be solved by molecular replacement. EhCoacto was expressed in minimal media containing selenomethionine (SeMet) for incorporating SeMet to protein. The SeMet derivative protein crystals diffracted to 1.5Å at selenium peak wavelength. The crystals belonged to P6 space group with unit cell parameters, $a=b=76.59\text{\AA}$, $c=54.649\text{\AA}$, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$. AutoSol and AutoBuild of Phaser could identify the heavy atom positions and build upto 60% of the model in P6₅ space group. This model was used for molecular replacement to solve the native EhCoacto structure at 1.4Å. The structure is almost completely refined with R and freeR values of 19% and 21% respectively. Further refinement is under progress. The structure shows the protein to be rich in β sheets and is similar to Human coactosin like protein (CLP).

Actin binding assay show that full length and C-term deleted Coactosin is able to bind actin filament. Actin depolymerisation kinetics shows that in higher concentration of Co actosin instead of depolymerising it stabilizes the actin filament. This protein has been shown to be associated with phagocytic cup formation in *E. histolytica*.

Establishment of a transfection system for *Entamoeba invadens*

Nozomi Mase^{1,2}, Kumiko Nakada-Tsukui¹, Tomoyoshi Nozaki^{1,2}

¹Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; ²Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan.

Trophozoites of *Entamoeba invadens*, which infect reptiles, serve as a model system in studying how differentiation occurs in *Entamoeba* due to their ability of in vitro stage conversion. Although encystation and excystation are essential processes for the pathogenicity and disease transmission of *Entamoeba* species, transfection systems is not yet available for this model organism *E. invadens*. In this study, we have established a transient transfection system for *E. invadens* for understanding the molecular mechanism behind differentiation.

To select appropriate genetic elements required for the expression of foreign genes, the transcriptional promoter and RNA processing activity of the 5'- and 3'-untranslated regions of actin (EiActin), cysteine synthase (EiCS), and protein disulfide isomerase (EiPDI) genes were examined using episomal reporter plasmids containing Renilla luciferase gene flanked by the 5'- and 3'-untranslated regions of the above-mentioned genes. Transfection was performed by lipofection, followed by luciferase assay. The flanking regions of the two of three genes, EiActin and EiCS gave detectable luciferase gene expression in *E. invadens*. Next, we optimized conditions for lipofection, such as DNA amount, co-incubation time with the DNA-lipofectamine mixture, and incubation time after transfection, using EiActin upstream and downstream regions. We found that 24-hour co-incubation with 5 ugof DNA-lipofectamine mixture and luciferase assay at 24 hours post-transfection resulted in the highest transfection efficiency for *E. invadens*. We are currently working on tetracycline-inducible expression system and stable transfection using neomycin and puromycin resistance genes.

Toll-like Receptors genetic polymorphism and Amoebic Liver Abscess in Mexico

Oswaldo Partida-Rodríguez¹, Eric G. Hernández Hernández¹, Carmen Maldonado Bernal², Miriam E. Nieves-Ramírez¹, Olivia Valenzuela Antelo³, Ulises Magaña Nuñez³, Edgar A. Rascón Castelo³, Valeria Zermeño León¹, Tobias Portillo Bobadilla¹, Liliana Rojas Velázquez¹, René Cerritos Flores¹, Alicia Valadez Salazar¹, Enrique González Rivas¹, Patricia Morán Silva¹, Cecilia Ximénez García¹.

¹Department of Experimental Medicine, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Mexico City. ²Laboratory of Hematology, Children's Hospital of Mexico Federico Gomez (HIM). ³Department of Chemistry and Biological Sciences, University of Sonora (UNISON), Hermosillo, Sonora.

The major Pattern Recognition Receptors are glycoproteins with leucine-rich repeats called Toll-Like Receptors (TLRs). TLR-mediated binding of *Entamoeba histolytica* molecular patterns provokes a series of intracellular signaling that leads to the activation of the innate immune system. TLR genes have single nucleotide polymorphisms (SNPs) that may influence their activity, and have been associated with disease susceptibility. We studied the associations of TLRs SNPs with susceptibility to develop amoebic liver abscess (ALA) by *Entamoeba histolytica* in Mexican individuals from the State of Sonora and from Mexico City (DF). We typed by allelic exclusion the SNPs R677W and R753Q of TLR2, D299G and T399I of TLR4, and -1237T/C and 2848G/A of TLR9. We compared SNPs frequencies of ALA patients (Sonora n=32, DF n=32) with control individuals (Sonora n=58, DF n=21). Frequencies in TLR2 and TLR4 SNPs in ALA patients were not statistically different when compared with controls. The allele T of TLR9 SNP -1237T/C in patients of ALA had frequencies of 95.3% in Sonora and 93.8% in DF, with similar proportions in controls. The allele G of SNP 2848G/A had frequencies of 40.0% and 55.0% in patients from Sonora and DF respectively, with no differences with controls. However, when the genotypes were analyzed, the genotype G/A in ALA patients from DF showed a statistically (p=0.008) increased frequency (63.3%) when compared with controls (25.0%), with an OR of 5.18 (95% CI 1.27-22.37). We will increase sample size in order to find out if the TLR9 2848G/A can be considered as a marker of susceptibility to ALA in population from the center of Mexico.

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CUTANEOUS AMOEBIASIS: the importance of molecular diagnosis in an emerging parasitic disease.

Patricia Morán¹, René Cerritos¹, Liliana Rojas¹, Valeria Zermeño¹, Alicia Valadez¹, Miguel Angel Reyes², Griselda Montes de Oca³, Enrique González¹, Oswaldo Partida¹, Eric Hernández¹, Miriam Nieves¹, Tobías Portillo¹, Manuel Ramiro³, Cecilia Ximénez¹.

¹Department of Experimental Medicine, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, Mexico, ²Urology Department, General Hospital of Mexico, Ministry of Health, Mexico City; ³Dermatology Department, General Hospital of Mexico, Ministry of Health, Mexico City; ⁴Unit of Education, Scientific Research and Health Policy Direction of Medical Benefits, Mexican Institute of Social Security Mexico City, Mexico.

Cutaneous amoebiasis is the least common clinical form of human amoebiasis. Cases usually correspond to patients with deep skin lesions in areas such as; anal, perineal, vulvae, vaginal and genitalia, as well as the diaper area in babies with intestinal diarrhea due to *E. histolytica* infection. In adults the most common location of cutaneous amoebiasis is the abdominal area as a consequence of the abdominal wall spontaneous involvement of amoebic liver abscess or as a complication of an amoebic liver abscess drainage procedure. In Mexico this pathology was occasionally observed before the late 1980's. However, in the last decades, most of the documented cases of this condition around the world are among sexually transmitted amoebiasis cases. The cases refereed in the present work are of sexually transmitted genital amoebiasis. The molecular characterization of the *Entamoeba* species present in the affected tissues allows not only the deferential diagnosis of disease, but the documentation for the first time of a case of cutaneous amoebiasis where *E. histolytica* and *E. dispar* was detected. This finding underline the importance of an opportune etiological diagnosis using specific and sensitive techniques to avoid the rapid destruction of tissues and the irreversible sequels on the anatomy and function of affected organs. On the other hand, the detection of a new evidence of mixed infections (*E. histolytica* and *E. dispar*) in an invasive case of amoebiasis open new perspectives in the study of the extraordinary complex host-parasite relationship in amoebiasis.

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Multiplex PCR assays for detection of mixed infection of *Entamoeba histolytica* and non-pathogenic *Entamoeba moshkovskii* or *Entamoeba dispar* in clinical specimen

Zulhainan Hamzah¹, Songsak Petmitr², Mathirut Mungthin³, Porntip Chavalitsheewinkoon-Petmitr¹

¹Department of Protozoology, ²Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University; ³ Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand

Because of an identical morphology both in cyst and trophozoite stage of three human *Entamoeba* species; *Entamoeba histolytica*, *Entamoeba moshkovskii* and *Entamoeba dispar*, they cannot be differentiated by a microscopic method. Development of molecular diagnosis of mixed infection of pathogenic *Entamoeba histolytica* and the other two non-pathogenic *Entamoeba* spp; *Entamoeba moshkovskii* and *Entamoeba dispar* is basically needed for clinical treatment. In this study, PCR assays were developed for detection and differential diagnosis of pathogenic amoeba; *Entamoeba histolytica* from non-pathogenic amoeba which may be found in humans such as *Entamoeba dispar* and *Entamoeba moshkovskii*. Each duplex PCR assay utilizes one forward primer derived from the middle of the small-subunit rRNA gene, whereas three reverse primers were designed from signature sequences specific to each of these three *Entamoeba* species. PCR generates 166-bp product with *E. histolytica* DNA, a 752-bp product with *E. dispar* DNA and a 580-bp product with *E. moshkovskii* DNA. Specificity and cross reaction test with different sources of pathogens, including bacteria and protozoa confirming the high specificity of the assay developed. A total of thirty clinical specimens were examined and *Entamoeba* species were successfully detected and differentiated using this assay. PCR assays are able to detect as low as 0.156 ng and 0.019 ng for mixed infection of *E. moshkovskii* and *E. histolytica*, respectively and 0.019 ng for mixed infection of *E. histolytica* and *E. dispar*. The economy of consumables and labour represented by this multiplex PCR assay greatly facilitates the diagnosis of a large number of samples, making it a rapid, cost-effective and appropriate tool for field epizootiological surveys.

Purification of ferredoxin isoforms of *Entamoeba histolytica* and their in silico homology modeling and protein-ligand interaction

Vahab Ali¹, Pushpendra Patel¹, Shadab Anwar¹, Rajesh Roushan³, Asmita Kumari¹, Pradeep Das², and Tomoyoshi Nozaki⁴

¹Laboratory of Molecular Biochemistry and Cell Biology, Dept. of Biochemistry, ²Dept. of Molecular Biology, RMRIMS, Agamkuan, Patna; ³Dept. of Pharmacoinformatics, NIPER, Hajipur; ⁴Dept. of Parasitology, NIID, Tokyo, Japan

Entamoeba histolytica is a unicellular amitochondriate protozoan parasite responsible for approximately 50,000 deaths occur annually world-wide. *Entamoeba histolytica* ferredoxin is an iron-sulfur protein which exploits the redox properties of their cofactors to transfer electrons in a wide variety of biochemical reactions. Knowing the manner of protein interaction is vital for understanding biological events. It donates an electron to and activates metronidazole, the most effective antiamebic drug. Ferredoxin genes were cloned, expressed and polyclonal antibodies were produced against purified ferredoxin isoforms. The knowledge of 3D structure of EhFdx can help to assist in rational design of anti-amoebic drugs. It has been shown that EhFdx are more likely to contain 4Fe-4S cluster. In general ferredoxin proteins are acidic in nature but *E. histolytica* posses one of the unusual isoform which is basic in nature (predicted pI-8.26). Homology model of ferredoxin were constructed using homologous structures as template for the model building, using SCHRODINGER 9.1/PRIME 2.1 software, the lowest energy of ferredoxin model was then assessed for stereo-chemical quality and side chain environment by PROCHECK, Ramachandran plot, ProSA2003 ver.4.0 and RMSD that showed the refined models are reliable. Further active site optimization of the ferredoxin model was performed by SiteMap (SCHRODINGER9.1). 5-nitroimidazole derivatives, Nitazoxamide and their metabolite, ferredoxin inhibitors were docked in to the model and model-ligand complex were used to validate the active site architecture. Structurally and functionally important residues were identified by subsequent characterization of the secondary structure, which suggesting that one of the ferredoxin isoform interact with metronidazole compare to other isoforms. It seems that other ferredoxin isoforms have different functions.

Interaction of *E. histolytica* with bacteria in the pathogenic processes of the liver abscess: an ecological and evolutionary story.

Valeria Zermeño, Cecilia Ximénez, Wendy Flores, Miguel Romero, Patricia Moran, Alicia Valadez, René Cerritos.

Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, México DF, México.

Liver abscess is defined as the accumulation of pus and /or necrotic material within the liver, caused by bacteria (pyogenic) and/or the protozoan *Entamoeba histolytica* (amoebic). Many clinical features has to be considered when the differential and etiologic diagnosis and treatment has to be established: demographic risk factors, recent travels to endemic regions of amoebiasis, previous diarrhea events, recent traumas, elevated leukocyte count, blood cultures and positive serology to amoebic antigens. Nevertheless molecular tools are not rutinary considered to confirm etiological diagnosis. In this study, we analyzed a group of 49 abscess samples using PCR, from patients clinically diagnosed as pyogenic (24) or amoebic (25). Results of PCR amplification of not coding intergenic short tandem repeated sequences associated to tRNA genes showed the presence of amoebic sequences in 44 of analyzed samples. We also analyzed 29 (29/49) samples, for the presence of bacteria using the 16S rRNA as molecular marker. The presence of 16S rRNA was detected in 28 (28/29) of studied samples; subsequently the PCR products of 4 samples were cloned to finally sequence 80 clones of 16S rRNA in order to establish the taxonomic group of detected bacteria. In addition, bioinformatics analyses were performed to identify the frequency of genetic horizontal transference from bacteria to *E. histolytica* DNA, mainly of those genes involved in pathogenic processes. This work makes clear that the great majority of liver abscesses are not produced by only one infectious agent. On the contrary, the presence of amebas and bacteria together, within the liver, suggests that there is a close interaction between *E. histolytica* and certain bacterial groups in the pathogenesis strengthen and the evolution of microorganisms.

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Subcellular protein profiles of *Entamoeba histolytica* compared to *Entamoeba moshkovskii*

Saengduen Moonsom¹, Kanthinich Thima¹, Narumol Khomkhum¹, Somphob Leetachewa², Sittiruk Roytrakul³, Seiki Kobayashi⁴, Tomoyoshi Nozaki⁵ and Porntip Petmitr¹

¹Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, ²Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand, ³Genome Institute, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand, ⁴Department of Tropical Medicine and Parasitology, Keio University, Tokyo, Japan, ⁵Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan

The protozoan *Entamoeba histolytica* infects millions of people worldwide and causes amoebic dysentery, and amoebic liver abscess. *E. moshkovskii* is longtime considered as a free living amoeba, however recently reported as a human pathogen in some endemic countries. *E. histolytica* and *E. moshkovskii* are morphologically identical in both their trophozoites and cysts that make them are unable to be differentiated by the conventional microscopy. To search for phenotypic difference, cells were separated into cytosolic, membrane, nuclear and cytoskeletal fractions. Preliminary, SDS-PAGE showed difference in the protein profiles between these two species. Particularly, the 38-50 kDa and proteins which sizes are larger than 170 kDa were found mainly in the membrane, and nuclear/cytoskeletal fractions of *E. histolytica*, respectively. It is possible that these proteins may useful in further development of differential diagnosis or explanation of pathogenic mechanism of the two organisms. Protein identification will be performed by liquid chromatography-mass spectrometry.

Calmodulin like calcium binding protein EhCaBP3 Involved in Phagocytosis of the Protozoan Parasite *Entamoeba histolytica*

Saima Aslam¹, Narendra Padhan¹, Sudha bhattacharya² and Alok Bhattacharya¹

¹School of Life Sciences, Jawaharlal Nehru University New Delhi-110067, India

² School of Environmental Sciences, Jawaharlal Nehru University, New Delhi-110067, India,

Entamoeba histolytica, a protozoan parasite, is a causative agent of invasive amoebiasis and it is third largest killer after malaria and schistosomiasis among parasites. Calcium signaling plays a crucial role in the pathogenesis of several protozoan parasites including *E. histolytica* and is mediated through a number of calcium binding proteins (CaBPs). In order to understand the molecular mechanisms of pathogenesis we have identified several CaBPs in our laboratory and are trying to decipher their role in calcium signaling network. Out of 27 CaBPs of *E. histolytica*, two have been characterized by us previously. In this study we have characterized EhCaBP3. It is a multi EF-hand Calcium binding protein with high affinity for calcium and also shows highest sequence similarity of all amebic proteins to calmodulin. A specific antibody was raised against the recombinant EhCaBP3 and was used to visualize distribution of this protein in *E. histolytica* during proliferation and red blood cell phagocytosis. Immunofluorescent data suggested that it may be involved in phagocytosis as it was found enriched in phagocytic cups and also in early phagosomes. Live cell imaging showed that EhCaBP3 accumulated within 14s at the site of RBC attachment and that it later moved towards the tip of the cups before closure. A Ca^{2+} -insensitive mutant was generated by site directed mutagenesis of key residues of all EF hands. Overexpression of this mutant in trophozoites showed that there was a decrease in RBC uptake compared to the controls, suggesting that Ca^{2+} -EhCaBP3 is required for phagocytosis. Confocal microscopy revealed that both wild type and mutant proteins interact with actin. Downregulation of EhCaBP3 by anti-sense RNA showed that there was a delay in phagocytic cup formation and there was also a defect in closure of cup, suggesting that EhCaBP3 is required both in the initiation as well as phagosome formation. Surprisingly EhCaBP3 was also found in the nucleus in addition to the cytoplasm. It is not clear how it reaches nucleus as it does not have nuclear localization signal. Overall it appears that EhCaBP3 is not a homolog of calmodulin and has an important role in amebic biology.

Seroprevalence of *Entamoeba histolytica* in the context of HIV and AIDS: the case of Vhembe district, in South Africa's Limpopo province

Samie A¹, Barrett LJ², Bessong PO¹, Ramalivhana JN¹, Mavhandu LG¹, Njayou M³ and Guerrant RL²

1. AIDS Virus Research Laboratory, Department of Microbiology, University of Venda Private Bag X5050, Thohoyandou 0950, Limpopo Province, South Africa
2. Center for Global Health, Carter–Harrison Building, MR-6, Room 2520, 345 Crispell Drive/P.O. Box 801379, University of Virginia, Charlottesville, VA 22902-1379, U.S.A.
3. Laboratory of Microbial Biotechnology, University of Yaoundé 1, P.O. Box 2001, Yaoundé, Cameroon

Entamoeba histolytica is a protozoan parasite that causes amebic dysentery and liver abscess. The disease is common in tropical regions of the world where hygiene and sanitation is often approximate. With the advents of HIV and AIDS, several organisms have been identified as potential opportunistic pathogens. However, it is not clear whether amoebiasis is an opportunistic infection or not. In a recent study in northern South Africa, the seroprevalence of *Entamoeba histolytica* infection among 257 HIV positive and 117 HIV-negative individuals was determined, using an ELISA for the detection of antibodies reacting with the parasite's galactose/–acetyl-D-galactosamine (Gal/GalNAc)-inhibitable adherence lectin. Overall, 34.0% of the 374 participants (36.1% of the females and 28.1% of the males) were found seropositive for *E. histolytica*. Although all age-groups were affected by the amoebic pathogen, the subjects aged 50–59 years had the highest seroprevalence (69.2%). The seroprevalence of *E. histolytica* was also significantly higher among the HIV-positive subjects than among the HIV-negative (42.8% vs. 14.5%; $\chi^2=8.65$; $P=0.0001$). Among the HIV-positive subjects, those with fewer than 200 CD4 cells/ μ l were relatively more likely to be seropositive for *E. histolytica* (60.3% vs. 43.8%; $\chi^2=4.016$; $P=0.045$). This is the first report indicating a positive association between *E. histolytica* infection and HIV in South Africa. Further studies, for example to determine the occurrence of diarrhoea or liver abscess in the study area, in relation to seropositivity for *E. histolytica* and/or HIV, are now needed.

Accumulation of pre ribosomal RNA and its processing intermediates in *Entamoeba invadens* during encystation

Sandeep Ojha¹, Alok Bhattacharya², Sudha Bhattacharya¹

¹School of Environmental Sciences, JNU, New Delhi, India

²School of Life Sciences J.N.U., New Delhi

Transcription of rRNA genes is highly regulated to respond to both general metabolism and stress conditions. Low glucose and nutrient deficiency in medium is a kind of stress that leads to encystation in *Entamoeba*. The ribosomal RNA genes in *E.invadens* (model to study encystation) are located exclusively on extra chromosomal circles (22.4kb). Each circle encodes a single transcription unit. We mapped the transcription start site of the rDNA unit which is 1782 bp upstream to the 5' end of 18SrRNA. Using 5'ETS probe and northern hybridization we measured the level of pre rRNA (7.8Kb) in cells induced to encyst in low glucose (LG medium) . Pre rRNA and its processing intermediates went down for the first 24 hrs in LG medium and started accumulating after that up to 72hrs (mature cysts form within 72 hrs in LG medium). Further we looked at the transcripts of selected ribosomal protein genes and we found that transcript of these genes go down for the first 16 hrs and start accumulating up to 48 hrs and after which the level drop again. We are doing nuclear run-on and pulse chase experiments to understand the dynamics of pre rRNA and assembly into ribosomes during encystation.

Crystal Structure of CaBP5 from *Entamoeba histolytica*

Sanjeev Kumar^{1,2}, Rana Zaidi² and S.Gourinath¹

1. School of Life Sciences J.N.U., New Delhi

2. Department of Biochemistry, Jamia Hamdard University, New Delhi

Entamoeba histolytica is the etiological agent for human amoebic colitis and liver abscess and causes a high level of morbidity and mortality worldwide, particularly in developing countries. There are a number of studies that show the involvement of Ca^{2+} and its binding proteins in amoebic pathogenesis. Towards investigation of structure and function of calcium binding proteins in *Entamoeba histolytica*, we have cloned CaBP5 from this organism. CaBP5 has been overexpressed and purified by Ni^{2+} -NTA affinity followed by gel filtration chromatography. Crystals of CaBP5 were grown in the presence of 2.6-2.8 M sodium acetate at pH 7.0 with 10 mM MgCl_2 . Further selenomethionine derivative proteins were made to overcome phase problem. Crystals was diffracted at 1.9Å resolution to ESRF BM-14, data were indexed and processed in space group C2, crystals parameter were $a = 70.55$, $b = 44.45$, $c = 47.73$, $\alpha = 90.0$, $\beta = 108.93$, $\gamma = 90.0$. About 50% residues were build by auto building program (Arp/Warp), rest of residues were traced manually in COOT, refinement was done by Refmac5. The structure of EhCaBP5 has been refined to R and R_{free} 20 and 24. Refined structure contains 131 residues and six residues were missing from n-terminal. Six His tag residues were also traced. The structure shows the presence of one molecule in the asymmetric unit. The structure is rich in α helices and two small stretch of antiparallel β sheet. Out of two EF-hands motifs only one is found with bound calcium. The overall structure is entirely different from the Calmodulin which is 29% identical to EhCaBP5. Further biophysical characterization is under process.

Identification of proteins targeted by cytosolic thioredoxin in *Entamoeba histolytica*

Sarah Schlosser, David Leitsch and Michael Duchêne

Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria

Entamoeba histolytica, an intestinal protozoan that is the causative agent of amoebiasis, possesses a functional NADPH-dependent thioredoxin system comprising the dithiol-containing redox proteins thioredoxin (Trx) and thioredoxin reductase (TrxR). Both proteins were found to be covalently modified by the 5-nitroimidazole drug metronidazole which consequently led to the loss of disulfide reducing activity of the TrxR/Trx system and the covalent modification of only a few defined proteins. The aim of the present study was to search systematically for further interaction partners of thioredoxin in order to extend our knowledge about the lethal action of metronidazole in *E. histolytica*. Based on the Trx reduction mechanism we constructed an active site mutant of Trx lacking the resolving cysteine residue. The recombinant mutant protein (EhTrx^{C34S}) was immobilized on Ni-NTA resin to capture target proteins from *E. histolytica* cell extracts after formation of intermolecular disulfide bonds. EhTrx^{C34S} and covalently linked proteins were eluted and visualized by two-dimensional gel electrophoresis and Coomassie Blue staining. Nineteen out of 55 Trx-captured proteins were analyzed by liquid chromatography-tandem mass spectrometry yielding 14 putative Trx binding partners which can be classified in five categories corresponding to definite biological processes: metabolism, detoxification and defense, protein folding and degradation, cytoskeleton and vesicular trafficking. Besides NADPH-dependent alcohol dehydrogenase and 2-Cys peroxiredoxin, which are already known to interact with *E. histolytica* Trx, we could identify new proteins that had not previously been associated with redox-mediated regulation in *E. histolytica* such as serine acetyltransferase-1 which catalyzes the O-acetylation of serine, the first reaction in the two-step process of cysteine biosynthesis, as well as aminoacyl-histidine dipeptidase which belongs to metallopeptidase family M20 and catalyzes the cleavage and release of an N-terminal amino acid from an Xaa-His dipeptide. The interaction of Trx with both enzymes and its influence on the enzymatic activity will be studied in further detail.

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Purification, characterization, and homology modeling of EhNbp and EhCfd proteins involved in Fe-S clusters assembly in *E. histolytica*

Shadab Anwar¹, Pushpendra Patel¹, Rajesh Roushan², Pradeep Das³, Tomoyoshi Nozaki⁴ A.K Roy⁵, and Vahab Ali¹

¹Dept. of Biochemistry, ³Dept. of Molecular Biology, RMRIMS, Agam Kuan, Patna; ²Dept. of Pharmacoinformatics, NIPER, Hajipur; ⁴Dept. of Parasitology, NIID, Tokyo Japan; ⁵PG Dept. of Biotechnology, TMB University, Bhagalpur

E. histolytica is a unicellular amitochondriate protozoan parasite causing bloody diarrhea/amoebiasis or liver abscess. Iron sulfur clusters are ubiquitous and versatile protein cofactor used for catalysis, electron transfer, gene regulation and iron sensing in a living cell. These simple inorganic cofactors are synthesized and inserted into apo-proteins by complex protein machineries i.e. NIF, SUF and ISC assemblies including cytosolic as well as export machinery. *E. histolytica* possess the NIF system and three homologs of the CIA (Cytosolic Fe-S cluster Assembly), annotated as Nbp, Cfd and Nar proteins are involved in the Fe-S cluster assembly and maturation of Fe-S proteins in the cytoplasm. Both EhNbp and EhCfd genes cloned in pET-15b vector and expressed in *E. coli* host as fusion protein with histidine tag. The expressed proteins were purified by affinity column chromatography using Ni-NTA agarose. Polyclonal antibodies were produced in rabbit against both proteins. Homology models of EhNbp, EhCfd proteins were constructed using homologous crystal structures as template that have the highest sequence homology with the target protein for the model building using the program Schrodinger9.1/prime2.1 in the optimized mode. Swiss-PDB Viewer 3.5 was also used to produce a structure based alignment and SWISS Model was used in the optimized mode to minimize energy. The final model was evaluated with PROCHECK and no residue was found in the disallowed regions of the Ramachandran plot. The total quality G- factor was close to zero, which is indicative of good quality model. These two proteins interact with each other and form stable complex which was identified by affinity column chromatography followed by western blot analysis. Cellular fractionation and immunofluorescence analysis showed cytosolic localization of these proteins.

Chitin biosynthesis pathway in encysting *Entamoeba*

Sintu Kumar Samanta and Sudip K. Ghosh

Department of Biotechnology, Indian Institute of Technology Kharagpur
Kharagpur -721302, India

Entamoeba histolytica causes amoebiasis which is transmitted through its cyst form. The thick chitin wall protects this cyst from the harsh environment outside human body. Though it is known that chitin is synthesized only in encysting *Entamoeba* but chitin synthesis pathway (CSP) is not yet well characterized. We have identified the chitin biosynthesis pathway genes from the *Entamoeba* Genome database and verified their expression profile at transcriptional level in encysting *Entamoeba invadens*. Semi-quantitative RT-PCR (sqRT-PCR) analysis has shown that all the chitin pathway genes are either entirely absent or transcribed at very low level in trophozoites. The mRNA expression of most of the CSP genes reached maximum during a period of 9-12 hr after in vitro initiation of encystation. The silencing of Glucosamine-6-P isomerase (Gln6Pi) reduced the chitin synthesis by nearly 50% that indicates that Gln6Pi might be the key enzyme in regulation of chitin synthesis in *Entamoeba*. Encystation also up regulates the expression of glycogen phosphorylase which is responsible for glycogen degradation. The chitin synthesis was significantly decreased (57%) in encysting cells treated with specific inhibitor against glycogen phosphorylase. So, it can be concluded that the glucose availed from stored glycogen degradation in trophozoites might be one of the major sources of glucose for chitin synthesis in *Entamoeba*.

***Entamoeba histolytica*/Entamoeba dispar/Entamoeba moshkovskii complex: An epidemiological study conducted among different tribes of Malaysian aborigines**

Tengku, S.A.¹, Hesham M. Al-Mekhlafi², Mohamed Kamel, A.G.³ and Norhayati, M.¹

¹Department of Parasitology and Medical Entomology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia.

²Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

³Department of Biomedical Science, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia.

Entamoeba histolytica, the causative agent of human amoebiasis is still a public health concern in Malaysia especially among aboriginal communities. A series of community-based surveys to determine the prevalence and risk factors associated with *E. histolytica*/*E. dispar*/*E. moshkovskii* complex (Entamoeba complex) infection were carried out among two different aboriginal tribes (Proto-Malay and Negrito) in selected villages in Negeri Sembilan and Perak, Peninsular Malaysia. Socioeconomic data were collected using a pre-tested questionnaire. Stool specimens were examined by formalin-ether concentration and trichrome stained techniques. Out of 289 participants, 8.7% of Proto-Malay (13/150) and 29.5% of Negrito (41/139) were found positive for Entamoeba complex infection. The prevalence of complex infection showed an age dependency relationship, with significantly higher rates observed among those aged less than 15 years. Multivariate analysis confirmed that did not washing hands after playing with soil or gardening was identified as significant risk factor of the infection for both tribes, while eating with hands was also observed as significant predictor for Proto-Malay. Further studies on molecular approaches are needed to distinguish the morphologically identical species of pathogenic, *E. histolytica* from the non-pathogenic, *E. dispar* and *E. moshkovskii*. As highlighted in this study, the dynamic of transmission is most probably due to improper hygienic practice such as hand washing. Reliable epidemiological data about the route of transmission is vital for effective implementation of control strategies on combating this infection among aboriginal population in Malaysia.

A novel de-N-acetylase from *Entamoeba histolytica*: Metal stimulated but not metal dependent

Mohammad Ashraf¹, Bhawna Yadav¹, Usha Yadav¹, Rohini Muthuswami¹, Sneha Sudha Komath¹.

School of Life Sciences, Jawaharlal Nehru University, New Delhi, India.

Entamoeba histolytica is a worldwide found parasitic protozoan which causes amoebiasis. This infection is highly endemic especially in developing countries like India. The first stage of infection is adherence to the target cells. Adherence to the target cell is mediated by GPI anchored Gal/GalNac lectin on its surface. Any defect in GPI anchor biosynthesis affects the virulence and pathogenesis of *E. histolytica*. GPI anchor biosynthesis occurs in the ER. Although GPI anchor biosynthesis pathway is conserved among all eukaryotes but there are species specific modifications at various steps. Therefore this pathway could be a potential therapeutic drug target. The first two steps takes place on the cytosolic side and rest of the steps occur inside ER lumen. N-acetylglucosaminylphosphatidylinositol formation at the first step is catalysed by a multisubunit enzyme, GPI-GnT. In the second step, the GlcNAcPI is de-N-acetylated by a single enzyme, PIG-L. PIG-L proteins from different species are known to have differences in their location, conformation as well as specificity of the active sites. These differences have been used to design species specific inhibitors such as those against trypanosoma and plasmodium. The active site of the PIG-L protein is cytosolic, so, it is easily targetable. Our study reports the detailed characterization of *E. histolytica* PIG-L. *E. histolytica* PIG-L protein is found to be optimally active at pH 5.5. The enzyme has an intrinsic low level of activity in the absence of metal but in presence of divalent cations, it is significantly stimulated. Metal binding induces a large conformational change in the protein that enhance the catalytic rates without altering the substrate affinity of the protein. To elucidate the mechanism of catalysis of the protein homology modeling and mutational analysis have been done.

Genotyping in *Entamoeba histolytica* based on polymorphism of SINE1 loci

Vandana Kumari¹, Lakshmi Rani Iyer², Jaishree Paul², Jaco J. Verweij³, C. Graham Clark⁴, Alok Bhattacharya² and Sudha Bhattacharya¹

¹ School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067, India

² School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

³ Department of Parasitology, Leiden University Medical Centre (LUMC), Netherlands

⁴ Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

Only 10% of the *Entamoeba histolytica* infections result in invasive disease. What determines the outcome of *E. histolytica* infection (from asymptomatic to colitis to liver abscess) is still a mystery, but one possibility is that it is linked to the genotype of the parasite. Here we describe a new method of genotyping based on the genomic distribution of SINE 1 elements. It is expected that the distribution pattern of SINE1 may vary among different strains of *E. histolytica*. The genomic location of all Eh SINE1 copies in strain HM-1:IMSS was determined by BLAST analysis. Primers were designed from the flanking sequence at each location and amplicons obtained from HM-1 :IMSS and Rahman strains were compared. Loci giving differential band pattern between these strains were analyzed in detail by Southern blotting to confirm the polymorphism, followed by cloning and sequencing of the locus from Rahman. Of two hundred fourteen loci analyzed, EhSINE1 was missing in four loci of Rahman. Three of these loci were suitable for strain identification and were tested using 7 other axenically cultivated *E. histolytica* strains and 16 clinical isolates. Five of eight possible combinations were encountered with these three loci along with new pattern which varied both from HM-1:IMSS and Rahman strains presumably due to sequence changes accompanying the SINE1 loss.

Association between genotype of *Entamoeba histolytica* with disease outcomes using PCR amplification of tRNA-linked short tandem repeats.

Virendra Jaiswal¹, Ujjala Ghoshal¹, Balraj Mittal², Uday C Ghoshal³

¹Departments of Microbiology, ²Department of Genetics, ³Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

Background: Genotype of *E. histolytica* may contribute to different outcomes of infection, pathogenesis of which is enigmatic. We evaluated whether tRNA-linked short tandem repeats (STRs) polymorphism is associated with outcome of infection. **Method:** Patients with amoebic liver abscess (ALA, n=107), dysentery (n=56) and asymptomatic cyst passers (n=24) were included. DNA was extracted (Qiagen mini kit) from stool (cyst passers and dysentery) and pus (ALA). DNA was amplified using six *E. histolytica* specific tRNA-linked STR primers (A-L5, D-A5, S^{TGA}-D, N-K2, R-R5 and S-Q). Genotypes were determined by PCR and STR sequence using six STR markers. **Results:** A total of 11 (I-1 to I-11), 18 (I-12 to I-29), and 24 (I-30 to I-53) genotypes were identified in cyst passers, dysentery and ALA, respectively. Among cyst passers, genotypes were I-1, I-2, I-4, I-5, I-9, I-11 (n=2 each), I-3 and I-6 (n=3 each), I-7 (n=4), I-8, and I-10 (n=1 each). Among dysenteric patients, genotypes were I-21 (n=7), I-25 (n=6), I-14, and I-26 (n=5 each), I-12 and I-20 (n=4 each), I-13, I-17, I-28 and I-29 (n=3 each), I-11, I-16, I-18, and I-19 (n=2 each), I-15, I-22, I-23, I-24, and I-27 (n=1 each). Among ALA patients, genotypes were I-28 (n=16), I-36 (n=12), I-11 (n=11), I-29 and I-37 (n=9 each), I-50 (n=7), I-49, I-51 (n=6 each), I-44 (n=4), I-35, I-39 (n=3 each), I-32, I-33, I-38, I-48 and I-53 (n=2 each), I-30, I-31, I-34, I-40, I-41, I-42, I-43, I-46, I-47, and I-52 (n=1 each). **Conclusion:** Genotypes of *E. histolytica* are different in patients with liver abscess, asymptomatic and dysentery patients.

Analysis of the targeting mechanism of mitosomal proteins in *Entamoeba histolytica*

Yoshitaka Murakami^{1,2}, Takashi Makiuchi¹, Tomoyoshi Nozaki^{1,3}

¹Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; ²Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan; ³College of Biological Science, University of Tsukuba, Tsukuba, Ibaraki, Japan.

Entamoeba histolytica possesses a reduced and highly divergent mitochondrion-related organelle called mitosome. We have previously shown that its primary function is sulfate activation. While the *E. histolytica* genome encodes only a few proteins that are known to be involved in the transport of mitosomal proteins and conserved in eukaryotic organisms, e.g., Tom40 and Sam50, the majority of mitosomal proteins apparently lack the canonical amino-terminal transit peptide. Thus, the mechanism of mitosomal transport in this organism remains totally unknown. We have examined potential mitosomal targeting domain(s) of ATP sulfurylase (AS), APS kinase (APSK), and inorganic pyrophosphatase (IPP), as well as other mitosomal proteins detected by proteomic analysis of the purified mitosomes. AS and IPP have notable features at the amino- and carboxyl-terminal regions, which we are examining whether or not being involved in the mitosomal transport. We will present the results of immunofluorescence analysis of the epitope-tagged truncated forms, lacking a potential targeting signal, of AS and IPP.

Decreased O-linked beta-N-acetylglucosamine levels on proteins accelerate human hepatoma cell death induced by protozoan parasite *Entamoeba histolytica*

Young Ah Lee¹, Kyoung-Ju Song¹, David Mirelman², Myeong Heon Shin¹

¹Department of Environmental Medical Biology, Institute of Tropical Medicine, and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea. ²Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

The enteric tissue-invasion protozoan parasite, *E. histolytica* leads to host cell apoptosis by the induction of various intracellular signal mechanisms in host cells. These modulations triggered by *E. histolytica* are closely associated with parasitic immune evasion mechanism as well as host defense. O-linked- β -N-acetylglucosamine (O-GlcNAc), a single sugar modification on the hydroxyl group of serine or threonine residues of nuclear and cytoplasmic proteins in eukaryotic cells. O-GlcNAcylation, similar to phosphorylation, has been thought to contribute the various cellular signal processes including apoptosis and proliferation. O-GlcNAc addition and removal are regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively. However, it is unknown whether *E. histolytica* can affect the O-GlcNAc level in host cells during Entamoeba-induced cell death. In this study, we investigated whether modulation of O-GlcNAcylated protein levels in host cells is involved in HepG2 cell death induced by *E. histolytica* using virulent strain HM-1, non-virulent Rahman. Co-incubation of HepG2 cells with HM-1 strain remarkably increased DNA fragmentation and LDH release compared to cells incubated with Rahman or medium alone. In addition, Entamoeba HM-1 induced tyrosine dephosphorylation and the cleavage of caspases-3 and calpain in HepG2 cells. Adherence of live HM-1 trophozoites caused the decrease of O-GlcNAcylated protein levels in HepG2 cells within 2 min. However, HepG2 cells incubated with Rahman retained O-GlcNAc levels on protein compared to cells incubated with HM-1. In addition, *E. histolytica*-induced deGlcNAcylation in HepG2 cells was dramatically reduced in cells pretreated with O-GlcNAcase inhibitors, PUGNAc or streptozotocin. In addition, DNA fragmentation and LDH release triggered by *E. histolytica* HM-1 were strongly inhibited by pretreatment of host cells with OGA inhibitor PUGNAc, suggesting that the deGlcNAcylation in host cell plays an important role in *E. histolytica*-induced HepG2 cell death. This study shows a potentially novel immune evasion mechanism to induce host cell death utilized by *E. histolytica*.

Regulation of phagocytosis by Rab8 and Rab7B in *Entamoeba histolytica*

Yuki Hanadate^{1,2}, Kumiko Nakada-Tsukui¹, Tomoyoshi Nozaki^{1,2}, and Yumiko Saito-Nakano¹

¹Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan, ²Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan.

Phagocytosis of host cells and bacteria is indispensable for the parasitization and pathogenesis of *Entamoeba histolytica*. The amoebic phagocytosis is initiated by the specific recognition and attachment of ligands via plasma membrane receptors. Engulfed particles are subsequently degraded by the specific hydrolases transported to newly formed phagosomes. Membrane traffic is responsible for the targeting of receptors to the plasma membrane, the internalization of receptor-bound prey, and the acidification and hydrolase recruitment of phagosomes. Rab GTPases are known to participate in membrane docking and fusion in the elaborately-regulated membrane traffic. We have previously identified 14 Rab proteins that are localized by the phagosomal proteome analysis of the phagosomes isolated with phagocytosed carboxylated latex beads. In this study, targeted epigenetic silencing was used to examine the physiological roles of phagosomal Rab proteins in phagocytosis. Among 5 phagosomal Rab genes tested, gene silencing of two Rab genes, e.g., Rab8 and Rab7B, caused remarkable reduction in phagocytic efficiency. Interestingly, repression of Rab8 expression showed defects in phagocytosis of erythrocytes, bacteria, and carboxylated latex beads, whereas repression of Rab7B expression showed defect in phagocytosis of both erythrocytes and bacteria, but not beads. These data support the premise that the amoeba utilizes prey-dependent pathways of phagocytosis. In addition, these results also indicate that Rab8 and Rab7B play important and distinct roles involved in a not-yet-identified process: e.g., the internalization or recycling of membrane receptors.

Late Arrivals

Evolutionary studies of food-borne zoonotic trematode flukes with reference to rDNA ITS-2 sequences and their secondary structures

Ranjana Sahu and Veena Tandon

Department of Zoology, North-Eastern Hill University, Shillong 793022, Meghalaya, India

Trematodes are a group of parasites infecting molluscs and vertebrates. Some of them are of zoonotic importance, infecting both humans and animals, and causing health problem, as well as significant economic losses. Food-borne trematodiasis (FTD) is one of the neglected tropical diseases (NTDs), which in terms of global burden represent the fourth most important group of communicable diseases worldwide. The most important species implicated in FTD include liver flukes (*Fasciola gigantica*, *Clonorchis sinensis*, and *Opisthorchis* spp); lung fluke (*Paragonimus* spp) and intestinal flukes (*echinostomatid* *Artyfechinostomum*, *Fasciolopsis buski*). In the present study we aimed at comparing the molecular characterization of all these potentially zoonotic trematodes. For the purpose, we chose the nuclear ribosomal DNA internal transcribed spacer 2 (rDNA ITS2) region as the exemplary marker region that has proven usefulness in differentiating molecular characters at higher taxonomic levels (species/genus and above).

PCR amplified products of ITS-2 cistron of various isolates representing the afore- mentioned genera under four families were sequenced and the sequences obtained matched with the accessions earlier deposited in the Genbank. The sequence data were analysed using various bioinformatics tools. BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used for sequence homology analysis and phylogenetic trees were constructed using distance-based (Neighbour-Joining, Maximum likelihood) and Character-based (Maximum Parsimony) methods in MEGA 5 (Tamura et al., 2011).

The analysis results revealed that all the families under study are monophyletic in origin. To supplement the study, a molecular morphometrics analysis of the ITS-2 secondary structures was also carried out using Mfold (<http://mfold.rna.albany.edu>). The rRNA secondary structures (showing a typical 4-helix form) further validated the inference. From the above study we had tried to see the evolutionary relationship between the various zoonotic species which can be further evaluated for the study of their interrelationship.

Keywords

Trematode, Zoonosis, Food-borne trematodiasis, Internal transcribed spacer 2, Phylogenetic tree, ITS 2 secondary structure.

Morphological and molecular characterization: Trematode metacercarial spectrum prevailing in the commonly edible fish and crab species in Northeast India

Voleentina D. Athokpam, Donald B. Jyrwa, Sunila Thapa and Veena Tandon

Department of Zoology, North-Eastern Hill University, Shillong-793022

Crustacea-borne trematodoses, which are mainly transmitted through consumption of improperly cooked or raw crabs, affects a large section of population, particularly in Asian countries, thus eliciting a remarkable morbidity and causing serious damage to health. In addition to this, fish-borne trematode (FBT) infections affect the health of more than 18 million people around the world. In India, centring in several mountainous regions of North eastern India, the natives have the habit of consuming raw or improperly cooked fish or crabs that still sustain viable infective stages (metacercaria) of trematode flukes in the muscle tissue. The present study was undertaken to ascertain the spectrum of metacercarial diversity in the commonly edible freshwater fishes and crab species in the northeast Indian state of Manipur and to adjudge their zoonotic potential, if any. Commonly edible fishes belonging to 15 species from 12 localities and crabs belonging to 2 species from 11 localities across Manipur state were surveyed for the purpose. The host tissues were examined using artificial digestion method and screened under light microscope for recovery of the parasite stages, if any. Morphological identification of the recovered metacercariae was carried out using the standard protocols for light microscopy (LM) and scanning electron microscopy (SEM). The study revealed that the crab species (*Barythelphusa lugubris masoniana* and *Potamiscus manipuriensis*) were found infected with 4 different types of metacercariae representing the families Microphallidae and Troglotremitidae, whereas 3 species of fishes (*Heteropneustes fossilis*, *Channa punctatus* and *C. straitus*) harboured 5 different types of metacercariae belonging to families Clinostomidae and Diplostomidae. To support the morphological identification, polymerase chain reaction (PCR)-based molecular methods were employed using the second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA. The rDNA ITS2 sequence data thus generated were used for identifying the taxa and phylogenetic trees constructed to study their evolutionary rates using various bioinformatics tools. The details of the various analyses are discussed.

Keywords: Trematode, Metacercaria, Molecular characterization, ITS2 sequences, Fish, Crustacea, Manipur

Gene expression profile of *Entamoeba histolytica* analyzed by RNA Seq

Christian Weber^{1,2}, Mikael Koutero³, Marc Deloger^{1,2}, Marie-Agnès Dillies³, Jean-Yves Coppée³, Enedina Jimenez-Cardozo⁴, María del Pilar Crisóstomo⁴, Victor Alberto Maravelez⁴, Chung-Chau Hon^{1,2} and Nancy Guillén^{1,2}

¹Institut Pasteur, Unité Biologie Cellulaire du Parasitisme and ²INSERM U786,

³ Institut Pasteur, Genopole, Plate-forme Transcriptome et Epigenome, Paris, France

⁴ Children's Hospital Federico Gomez, Mexico City, Mexico

The complete genome sequence of *Entamoeba histolytica* has been determined. Although gene annotation is still incomplete we have new opportunities for the examination of gene expression profiles in parasites subjected to modified life conditions. To identify gene expression changes that may be associated with parasite environmental adaptation and survival during infection, we used a deep RNA sequencing approach. The experiment included three biological conditions with three biological replicates per condition, leading to a total of nine samples. Oriented single read libraries were prepared from purified polyA RNAs and were sequenced on a Illumina HiSeq 2000 according to a multiplexed design with three samples per lane. After quality control and trimming of adapter sequences, the reads (70 to 80 millions) were mapped on the genome and counted by gene. A statistical analysis was then performed in order to normalize the data and highlight genes that are differentially expressed between pairs of conditions.

Here we present the characterization of gene expression of *E. histolytica* HM1:IMSS strain, using highly virulent parasites isolated from hamster liver abscesses. The data were then compared with those from trophozoites from (i) the same strain converted to attenuated upon loss of their ability to form liver abscesses (following long-term axenic culture), (ii) with cultured trophozoites recently isolated from stools.

Our data showed that (i) for all cases the genome is transcribed at levels near to completion; (ii) there are two gene categories: some are expressed in a specific condition and others are expressed in all conditions; (iii) an extreme stress response is associated to virulence

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List of participants

<p>AHAMAD, Jamaluddin School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India j.ahamadjnu@gmail.com</p>	<p>BABUTA, Mrigya School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India mrigyajnu@gmail.com</p>	<p>DAR-DER, JI Research and Diagnostic Center Centers for Disease Control No. 6, Linsen S. Rd., Taipei 10050, Taiwan jidarder@cdc.gov.tw</p>
<p>ALI, ElBakri Medical Laboratory Technology Department College of Health Sciences University of Sharjah PO Box 27272 Sharjah United Arab Emirates ali_elbakri@hotmail.com</p>	<p>BHATTACHARYA, Sudha School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India sb@mail.jnu.ac.in</p>	<p>DEBNATH, Anjan Department of Pathology Sandler Center for Drug Discovery University of California, San Francisco Byers Hall, Box 2550 1700 4th Street, Room 509 San Francisco, CA 94158 anjan.debnath@ucsf.edu</p>
<p>ANKRI, Serge Department of Microbiology, Faculty of Medicine, Rappaport Institute, TECHNION, Efron Street, Bat Galim, Haifa, Isreal. sankri@tx.technion.ac.il</p>	<p>BHATTACHARYA, Alok School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India alok.bhattacharya@gmail.com</p>	<p>DUCHENE, Michael Division for Special prophylaxis & Tropical Medicine, University of Vienna, Kinderspitalgasse 15, Vienna 195, Austria Michael.duchene@univie.ac.at</p>
<p>ANWAR, Shadab Rajendra Memorial Research Institute of Medical sciences Lab. of Molecular Biochemistry and cell Biology Deptt.of Biochemistry Patna-800007 shadabanwar25@gmail.com</p>	<p>BRIEBA DE CASTRO, Luis Gabriel Laboratorio Nacional de Genomica para la Diversidad CINVESTAV-IPN Campus Guanajuato Irapuato, Gto. Mexico lgbrieba@ira.cinvestav.mx</p>	<p>GAURAV, Amit K School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India amitgaurav23@gmail.com</p>
<p>ASLAM, Saima School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India Saima.bt@gmail.com</p>	<p>BRUCHHAUS, Iris Bernhard Nocht Institute for Tropical Medicine Bernhard Nocht Str.74 20359 Hamburg, Germany bruchhaus@bni-hamburg.de</p>	<p>GILCHRIST, Carol A Assistant Professor of Research Division of Infectious Diseases and International Health University of Virginia School of Medicine PO Box 801365 Charlottesville, VA , USA carol_gilchrist@virginia.edu</p>

ATTAR, Salahuddin S Medicinal Chemistry Lab. Department of Chemistry, Jamia Millia Islamia, New Delhi - 110025 India. attar.jmi@gmail.com	CERRITOS, René F Universidad Nacional Autónoma de México renecerritos@gmail.com	GOURINATH, Samudrala School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India SGN9@hotmail.com
AZAM, Amir Department of Chemistry Jamia Millia Islamia Jamia Nagar - 110025 India Amir_sumbul@yahoo.co.in	CHADEE, Kris University of Calgary, Department of Microbiology and Infectious Diseases, 3330, Hospital Drive, Alberta, Canada T2N 4N1 kchadee@ucalgary.ca	KUMAR, Jitender School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India jitu.arya@gmail.com
GREWAL, Jaspreet S Department of Biochemistry, Bose Institute, Kolkata, India jaspreetgrewal15@gmail.com	HON CHUNG, Chau Cell Biology of Parasitism Unit INSERM U389, Institute Pasteur Paris, France bigchung@pasteur.fr	KUMAR, Nitesh School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India niteshjnu@gmail.com
GULLIEN, Nancy Cell Biology of Parasitism Unit INSERM U389, Institute Pasteur Paris, France nancy.guillen@pasteur.fr	IYER, Lakshmi R School of Life Sciences Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India lakshmiraniyer@gmail.com	KUMAR, Sanjeev School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India sanjeev.b23@gmail.com
GUPTA, Abhishek K School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India abhijnu.abhishek@gmail.com	JAISWAL, Virendra Department of Microbiology, SGPGIMS, Lucknow-226014, U.P., India.	KUMARI, Vandana School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India vandanajha15@gmail.com
HALL, Neil Director, Centre for Genomic Research Institute of Integrative Biology Biosciences Building Crown Street University of Liverpool Liverpool L69 7ZB neil.hall@liverpool.ac.uk	JEELANI, Ghulam Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan jeelani22@gmail.com	KYOUNG-JU, Song Department of Environmental Medical Biology, Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul, Korea kjsong261@yuhs.ac

<p>HANADATE, Yuki Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan yuki@nih.go.jp</p>	<p>JHINGAN, Gagan D. National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India gaganjhingan@gmail.com</p>	<p>LABRUYERE, Elisabeth Cell Biology of Parasitism Unit INSERM U389, Institute Pasteur Paris, France elabruye@pasteur.fr</p>
<p>HATAM, Gholamreza Department of Parasitology and Mycology School of Medicine Shiraz University of Medical Sciences Shiraz, Iran hatam908@yahoo.com</p>	<p>KANNATHASAN, Selvam Division of Parasitology, Department of Pathology, Faculty of Medicine, University of Jaffna, Sri Lanka selvamkannathasan@gmail.com</p>	<p>LEE, Young Ah Department of Environmental Medical Biology, Institute of Tropical Medicine and Brain Korea 21 Project for Medical Science Yonsei University College of Medicine Seoul, Korea afri00@naver.com</p>
<p>HERNANDEZ Eric G Laboratory of Immunology Department of Experimental Medicine General Hospital of Mexico School of Medicine National Autonomous University of Mexico + 52 55 5623 2671 ericghdz@yahoo.com.mx</p>	<p>KAUR Devinder School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India kaur.devbio@gmail.com</p>	<p>PATEL Pushpendra M. S. (Pharm) Biotechnology NIPER, Hajipur Bihar, India pushpendra.niper@gmail.com</p>
<p>LOTTER, Hannelore Bernhard- Nocht Institute for Tropical Medicine Department of Molecular Parasitology Bernhard-Nocht- Str.74 20359 Hamburg, Germany lotter @bni-hamburg.de</p>	<p>MUKHERJEE, K Avik Div. of Parasitology. National Institute of Cholera and Enteric Diseases. Indian Council of Medical Research (Govt. of India) Kolkata, India aavvik100@gmail.com</p>	<p>PAUL, Jaishree School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India jpaul33@hotmail.com</p>
<p>MANSURI, Shahid M School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India shahid581983@gmail.com</p>	<p>NAKADA-TSUKUI, Kumiko Department of Parasitology National Institute for Infectious Diseases Tokyo 162-8640, Japan kumiko@nih.go.jp</p>	<p>PENULIAR, Gil M. Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan gmp@nih.go.jp</p>

<p>MARUMO, Konomi Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan konomi@nih.go.jp</p>	<p>NAYLOR, Caitlin Division of Infectious Diseases and International Health University of Virginia Health System cgn5q@virginia.edu</p>	<p>PETMITR, Porntip Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchataewi, Bangkok tmppm@mahidol.ac.th</p>
<p>MATT, Julia Institute of Special Prophylaxis & Tropical Medicine, University of Vienna, julia.matt@meduniwien.ac.at</p>	<p>NOZAKI, Tomoyoshi Department of Parasitology National Institute for Infectious Diseases 1-231 Toyama, Shinjuku,ku Tokyo 162-8640, Japan nozaki@nih.go.jp</p>	<p>PETRI, William A. Jr. Chief, Division of Infectious Diseases and International Health University of Virginia Health System Carter Harrison Building Room 1709A 345 Crispell Drive P.O. Box 801340 Charlottesville VA 22908-1340 wap3g@virginia.edu</p>
<p>MEZA, Isaura Departamento de Biomedicina Molecular CINVESTAV-IPN Mexico DF, Mexico imeza@cinvestav.mx</p>	<p>NOZOMI, Mase Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan nozomi-m@nih.go.jp</p>	<p>RAHA, Sanghamitra Crystallography and Molecular Biology division, Saha Institute of Nuclear Physics, 1/AF Bidhan nagar, Kolkata-700064, Indiasanghamitra.raha@saha.ac.in</p>
<p>MIRELMAN, David Department of biological Chemistry Weizmann Institute of Science Rehovot, 76100, Israel david.mirelman@weizmann.ac.il</p>	<p>OJHA, Sandeep School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India sandy.mbt@gmail.com</p>	<p>TIWARI, Swati School of Biotechnology, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India swati_tiwari@mail.jnu.ac.in</p>
<p>REED, Sharon L. Microbiology and Virology laboratories UCSD Medical Center 200 W. Arbor Drive. San Diego CA 92103-8416, USA sreed@ucsd.edu</p>	<p>SHYLLA, Jollin Department of Zoology, North-Eastern Hill University, Shillong 793022, Meghalaya, India jolleneandrea@gmail.com</p>	<p>VERMA, K Anil School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India anilv228125@gmail.com</p>

<p>RODRIGUEZ, Oswaldo P Laboratory of Immunology Department of Experimental Medicine General Hospital of Mexico School of Medicine National Autonomous University of Mexico + 52 55 5623 2671 oswpartida@yahoo.com.mx</p>	<p>SINGH, Nishant School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India nishant900@gmail.com</p>	<p>WILSON, Ian Institute of Integrative Biology Biosciences Building Crown Street University of Liverpool Liverpool L69 7ZB Ian.Wilson@liverpool.ac.uk</p>
<p>SAHA, Arpita School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India arpitalively@gmail.com</p>	<p>SINGH, Upinder Department of Internal Medicine and Microbiology, Stanford University School of Medicine, S-141 Grant building 300 Pasture Drive Stanford CA 94305-5107, USA usingh@stanford.edu</p>	<p>XIMENEZ, G.Cecilia Laboratory of Immunology Department of Experimental Medicine General Hospital of Mexico School of Medicine cximenez@servidor.unam.mx</p>
<p>SAMANTA, Sintu K Department of Biotechnology, Indian Institute of Technology, Kharapur-721302, India samantasintu@gmail.com</p>	<p>SOMLATA School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India somlata83@gmail.com</p>	<p>YADAV, Usha School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India ushayadav16@gmail.com</p>
<p>SAMIE, Amidou Department of Microbiology University of Venda, Private Bag X5050 Thohoyandou 0950 samieamidou@yahoo.com</p>	<p>SRIVASTAVA, Ankita School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India ankita26@gmail.com</p>	<p>YADAV, Vijay P School of Environmental Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India viju.yadav@gmail.com</p>
<p>SATO, Emi Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan satoe@nih.go.jp</p>	<p>TENGKU, Anwar S Department of Parasitology and Medical Entomology Faculty of Medicine, University of Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia syahbyasree@yahoo.com</p>	<p>YOSHITAKA, Murakami Department of Parasitology National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan mrkmystk@nih.go.jp</p>
<p>SCHLOSSER, Sarah Institute of Special Prophylaxis & Tropical Medicine, University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria sarah.schlosser@meduniwien.ac.at</p>	<p>CLARK, Graham Department of Infectious and Tropical Diseases London School of Hygiene and Tropical Medicine Keppel Street, London WC1E 7HT, England, GB graham.clark@lshtm.ac.uk</p>	<p>WEBER, Christian Cell Biology of Parasitism Unit INSERM U389, Institute Pasteur Paris, France cweber@pasteur.fr</p>

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